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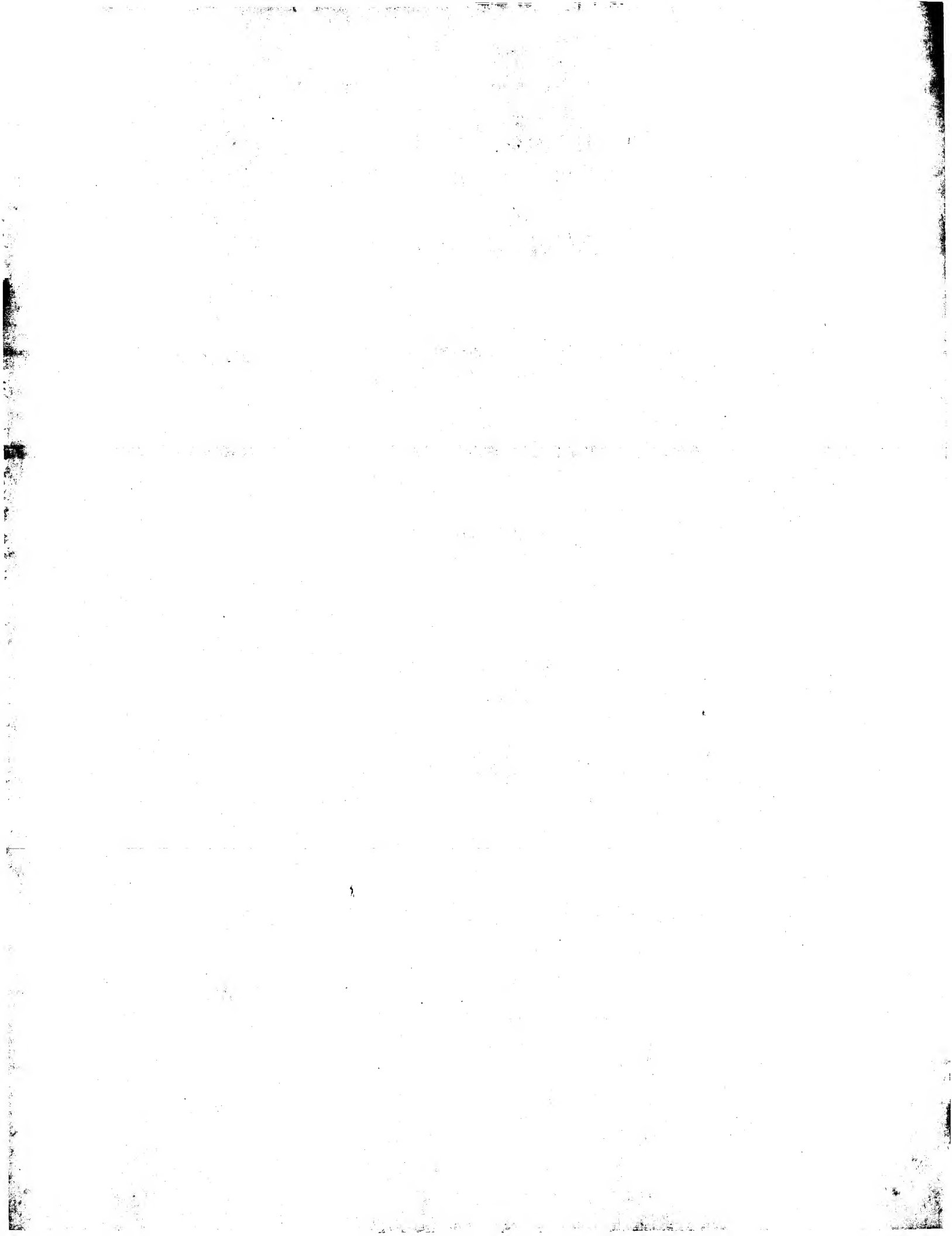
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶ : C12N 15/12, C07K 14/715, C12N 5/10, A01K 67/027, C07K 19/00, C12N 15/62, C07K 16/28, I/107, C12Q 1/68, G01N 33/50, 33/566, A61K 38/17, 48/00, C12N 1/21 // (C12N 1/21, C12R 1:19)</p>	A1	<p>(11) International Publication Number: WO 97/23614</p> <p>(43) International Publication Date: 3 July 1997 (03.07.97)</p>
<p>(21) International Application Number: PCT/US96/20621</p> <p>(22) International Filing Date: 20 December 1996 (20.12.96)</p> <p>(30) Priority Data: 08/577,788 22 December 1995 (22.12.95) US 08/706,945 3 September 1996 (03.09.96) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 08/706,945 (CIP) Filed on 3 September 1996 (03.09.96)</p> <p>(71) Applicant (for all designated States except US): AMGEN INC. [US/US]; Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US).</p> <p>(72) Inventors; and (73) Inventors/Applicants (for US only): BOYLE, William, J. [US/US]; 11678 Chestnut Ridge Street, Moorpark, CA (US). LACEY, David, L. [US/US]; 614 Paseo Vista, Thousand Oaks, CA (US). CALZONE, Frank, J. [US/US]; 841 Rim</p>		<p>Crest Circle, Westlake Village, CA (US). CHANG, Ming-Shi [US/US]; 736 Calle Las Colinas, Newbury Park, CA (US).</p> <p>(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>
<p>(54) Title: OSTEOPROTEGERIN</p> <p>(57) Abstract</p> <p>The present invention discloses a secreted polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily and is involved in the regulation of bone metabolism. Also disclosed are nucleic acids encoding osteoprotegerin, polypeptides, recombinant vectors and host cells for expression, antibodies which bind OPG, and pharmaceutical compositions. The polypeptides are used to treat bone diseases characterized by increased resorption such as osteoporosis.</p>		

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OSTEOPROTEGERIN

Field of the Invention

The invention relates generally to
5 polypeptides involved in the regulation of bone
metabolism. More particularly, the invention relates to
a novel polypeptide, termed osteoprotegerin, which is a
member of the tumor necrosis factor receptor
superfamily. The polypeptide is used to treat bone
10 diseases characterized by increased bone loss such as
osteoporosis.

Background of the Invention

Polypeptide growth factors and cytokines are
15 secreted factors which signal a wide variety of changes
in cell growth, differentiation, and metabolism, by
specifically binding to discrete, surface bound
receptors. As a class of proteins, receptors vary in
their structure and mode of signal transduction. They
20 are characterized by having an extracellular domain that
is involved in ligand binding, and cytoplasmic domain
which transmits an appropriate intracellular signal.
Receptor expression patterns ultimately determine which
cells will respond to a given ligand, while the
25 structure of a given receptor dictates the cellular
response induced by ligand binding. Receptors have been
shown to transmit intracellular signals via their
cytoplasmic domains by activating protein tyrosine, or
protein serine/threonine phosphorylation (e.g., platelet
30 derived growth factor receptor (PDGFR) or transforming
growth factor- β receptor-I (TGF β R-I), by stimulating
G-protein activation (e.g., β -adrenergic receptor), and
by modulating associations with cytoplasmic signal

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transducing proteins (e.g., TNFR-1 and Fas/APO) (Heldin, Cell 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, et al. Cell 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen et al., Chemistry 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNF α . (Goeddel et al. Cold Spring Harbor Symp. Quart. Biol. 51, 597-609 (1986); Nagata et al. Science 267, 1449-1456 (1995)). TNF α binds to distinct, but closely related receptors, TNFR-1 and TNFR-2. TNF α produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler et al. Ann. Rev. Biochem. 57, 505-518 (1988)).

TNF α is believed to mediate acute and chronic inflammatory responses (Beutler et al. Ann. Rev. Biochem. 57, 505-508 (1988)). Systemic delivery of TNF α induces toxic shock and widespread tissue necrosis. Because of this, TNF α may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda et al. Cell 75, 1169-1178 (1993)), is associated with autoimmunity (Fisher et al. Cell 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects of many disease states, which suggests that agents that

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neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-1 receptors, and antibodies that bind TNF α , have been tested for their ability to neutralize systemic TNF α (Loetscher et al. Cancer Cells 3(6), 221-226 (1991)). A naturally occurring form of a secreted TNFR-1 mRNA was recently cloned, and its product tested for its ability to neutralize TNF α activity in vitro and in vivo (Kohn et al. PNAS USA 87, 8331-8335 (1990)). The ability of this protein to neutralize TNF α suggests that soluble TNF receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR- bearing cells.

An object of the invention to identify new members of the TNFR super family. It is anticipated that new family members may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily which encodes a secreted protein that is closely related to TNFR-2. By analogy to soluble TNFR-1, the TNFR-2 related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

25 Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a fetal rat intestinal cDNA library. A full-length cDNA clone was obtained and sequenced. Expression of the rat cDNA in a transgenic mouse revealed a marked increase in bones density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by the cDNA is termed Osteoprotegerin (OPG) and plays a role in promoting bone accumulation.

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The invention provides for nucleic acids encoding a polypeptide having at least one of the biological activities of OPG. Nucleic acids which hybridize to nucleic acids encoding mouse, rat or human OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also provided. Preferably, OPG is mammalian OPG and more preferably is human OPG. Recombinant vectors and host cells expressing OPG are also encompassed as are methods of producing recombinant OPG. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

Methods of treating bone diseases are also provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone, and bone loss due to rheumatoid arthritis or osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising OPG nucleic acids and polypeptides are also encompassed.

25 Description of the Figures

Figure 1. A. FASTA analysis of novel EST LORF. Shown is the deduced FRI-1 amino acid sequence aligned to the human TNFR-2 sequence. B. Profile analysis of the novel EST LORF shown is the deduced FRI-1 amino acid sequence aligned to the TNFR-profile. C. Structural view of TNFR superfamily indicating region which is homologous to the novel FRI-1.

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Figure 2. Structure and sequence of full length rat OPG gene, a novel member of the TNFR superfamily. A. Map of pMOB-B1.1 insert. Box indicates position of LORF within the cDNA sequence (bold line). Black box indicates signal peptide, and gray ellipses indicate position of cysteine-rich repeat sequences. B, C. Nucleic acid and protein sequence of the Rat OPG cDNA. The predicted signal peptide is underlined, and potential sites of N-linked glycosylation are indicated in bold, underlined letters. D, E. Pileup sequence comparison (Wisconsin GCG Package, Version 8.1) of OPG with other members of the TNFR superfamily, fas (SEQ ID NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID NO:130); tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132); osteo (SEQ ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ ID NO:135); 41bb (SEQ ID NO:136).

Figure 3. PepPlot analysis (Wisconsin GCG Package, Version 8.1) of the predicted rat OPG protein sequence. A. Schematic representation of rat OPG showing hydrophobic (up) and hydrophilic (down) amino acids. Also shown are basic (up) and acidic (down) amino acids. B. Display of amino acid residues that are beta-sheet forming (up) and beta-sheet breaking down) as defined by Chou and Fasman (Adv. Enz. 47, 45-147 (1948)). C. Display of propensity measures for alpha-helix and beta-sheet (Chou and Fasman, *ibid*). Curves above 1.00 show propensity for alpha-helix or beta-sheet structure. Structure may terminate in regions of protein where curves drop below 1.00. D. Display of residues that are alpha-forming (up) or alpha-breaking (down). E. Display of portions of the protein sequence that resemble sequences typically found at the amino end of alpha and beta structures (Chou and Fasman, *ibid*). F. Display of

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portions of the protein sequence that resemble sequences typically found at the carboxyl end of alpha and beta structures (Chou and Fasman, *ibid*). G. Display of portions of the proteins sequence typically found in turns (Chou and Fasman, *ibid*) H. Display of the helical hydrophobic moment (Eisenberg et al. Proc. Natl. Acad. Sci. USA 81, 140-144 (1984)) at each position in the sequence. I. Display of average hydrophathy based upon Kyte and Doolittle (J. Mol. Biol. 157, 105-132 (1982)) and Goldman et al. (reviewed in Ann. Rev. Biophys. Biophys. Chem. 15, 321-353 (1986)).

Figure 4. mRNA expression patterns for the OPG cDNA in human tissues. Northern blots were probed with a 32P-labeled rat cDNA insert (A, left two panels), or with the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the OPG cDNA in hepatocytes. Northern blot expression of HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic mice. Panel A-F. Control Mice. G-J, OPG expressing mice. At necropsy, all animals were radiographed and photographs prepared. In A-F, the radiographs of the control animals and the one transgenic non-expressor (#28) are shown. Note that the bones have a clearly defined cortex and a lucent central marrow cavity. In contrast, the OPG (G-J) animals have a poorly defined cortex and increased density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG transgenic mice. A-D. Representative photomicrographs of bones from control animals. In A and B, low (4X, 10X) power

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images of the femurs are shown (Masson Trichrome stain). Stains for tartrate resistant acid phosphatase (TRAP) demonstrate osteoclasts (see arrows) both resorbing cartilage (C) and trabecular bone (D). Note the flattened appearance of osteoclasts on trabecular bone. E-H. Representative photomicrographs of bones from OPG-expressing animals. In E and F, low (4X, 10X) power images of the femurs are shown (Masson Trichrome stain). The clear region is the growth plate cartilage, blue stained area is bone, and the red area is marrow. Note that in contrast to the controls, the trabecular bone has not been resorbed resulting in the absence of the usual marrow cavity. Also, the resulting trabeculae have a variegated appearance with blue and clear areas. The clear areas are remnants of growth plate cartilage that have never been remodelled. Based on TRAP stains, these animals do have osteoclasts (see arrows) at the growth plate (G), which may be reduced in number. However, the surfaces of the trabeculae away from the growth plate are virtually devoid of osteoclasts (H), a finding that stands in direct contrast with the control animals (see D).

Figure 8. HE-OPG expressors do not have a defect in monocyte-macrophage development. One cause for osteopetrosis in mice is defective M-CSF production due to a point mutation in the M-CSF gene. This results in a marked deficit of circulating and tissue based macrophages. The peripheral blood of OPG expressors contained monocytes as assessed by H1E analysis. To affirm the presence of tissue macrophages, immunohistochemistry was performed using F480 antibodies, which recognize a cell surface antigen on murine macrophages. A and C show low power (4X)

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photomicrographs of the spleens from normal and CR1 overexpressors. Note that both animals have numerous F480 positive cells. Monocyte-macrophages were also present in the marrow of normal (B) and HE-OPG overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human OPG cDNA clones. A, B. Mouse cDNA and protein sequence. C, D. Human cDNA and protein sequence. The predicted signal peptides are underlined, and potential sites of N-linked glycosylation are indicated in bold. E, F. Sequence alignment and comparison of rat, mouse and human OPG amino acid sequences.

Figure 10. Comparison of conserved sequences in extracellular domain of TNFR-1 and human OPG. PrettyPlot (Wisconsin GCG Package, Version 8.1) of the TNFR1 and OPG alignment described in example 6. Top line, human TNFR1 sequences encoding domains 1-4. Bottom line, human OPG sequences encoding domains 1-4. Conserved residues are highlighted by rectangular boxes.

Figure 11. Three-dimensional representation of human OPG. Side-view of the Molescript display of the predicted 3-dimensional structure of human OPG residues 25 through 163, (wide line), co-crystallized with human TNF β (thin line). As a reference for orientation, the bold arrows along the OPG polypeptide backbone are pointing in the N-terminal to C-terminal direction. The location of individual cysteine residue side chains are inserted along the polypeptide backbone to help demonstrate the separate cysteine-rich domains. The TNF β molecule is aligned as described by Banner et al. (1993).

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Figure 12. Structure of OPG cysteine-rich domains. Alignment of the human (top line SEQ ID NO:136) and mouse (bottom line) OPG amino acid sequences

5 highlighting the predicted domain structure of OPG. The polypeptide is divided into two halves; the N-terminus (A), and C-terminus (B). The N-terminal half is predicted to contain four cysteine rich domains (labeled 1-4). The predicted intrachain disulfide bonds are

10 indicated by bold lines, labeled "SS1", "SS2", or "SS3". Tyrosine 28 and histidine 75 (underlined) are predicted to form an ionic interaction. Those amino acids predicted to interact with an OPG ligand are indicated by bold dots above the appropriate residue. The

15 cysteine residues located in the C-terminal half of OPG are indicated by rectangular boxes.

Figure 13. Expression and secretion of full length and truncated mouse OPG-Fc fusion proteins. A. Map

20 indicating points of fusion to the human IgG1 Fc domain are indicated by arrowheads. B. Silver stain of a SDS-polyacrylamide gel of conditioned media obtained from cells expressing either Fl.Fc (Full length OPG fused to Fc at Leucine 401) or CT.Fc (Carboxy-terminal truncated

25 OPG fused to Fc at threonine 180) fusion protein expression vectors. Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line. C. Western blot of

30 conditioned media obtained from Fl.Fc and CT.Fc fusion protein expression vectors probed with anti-human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line.

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Figure 14. Expression of human OPG in *E. coli*. A. Construction of a bacterial expression vector. The LORF of the human OPG gene was amplified by PCR, then joined to a oligonucleotide linker fragment (top strand is SEQ ID NO:137; bottom strand is SEQ ID NO:127), and ligated into pAMG21 vector DNA. The resulting vector is capable of expressing OPG residues 32-401 linked to a N-terminal methionine residue. B SDS-PAGE analysis of uninduced and induced bacterial harboring the pAMG21-human OPG - 32-401 plasmid. Lane 1, MW standards; lane 2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole cell lysate; lane 8, purified inclusion bodies obtained from whole cell lysate.

Figure 15. Analysis of recombinant murine OPG produced in CHO cells by SDS-PAGE and western blotting. An equal amount of CHO conditioned media was applied to each lane shown, and was prepared by treatment with either reducing sample buffer (left lane), or non-reducing sample buffer (right lane). After electrophoresis, the resolved proteins were transferred to a nylon membrane, then probed with anti-OPG antibodies. The relative positions of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 16. Pulse-chase analysis of recombinant murine OPG produced in CHO cells. CHO cells were pulse-labeled with ³⁵S-methionine/cysteine, then chased for the indicated time. Metabolically labeled cultures were separated into both conditioned media and cells, and detergent extracts were prepared from each, clarified,

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then immunoprecipitated with anti-OPG antibodies. The immunoprecipitates were resolved by SDS-PAGE, and exposed to film. Top left and right panels; samples analyzed under non-reducing conditions. Lower left and right panels; samples analyzed under reducing conditions. Top and bottom left panels; Cell extracts. Top and bottom right panels; Conditioned media extracts. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 17. Expression of OPG in the CTLL-2 cell line. Serum-free conditioned media from CTLL-2 cells and CHO-mu OPG [1-401] transfected cells was prepared, concentrated, then analyzed by non-reducing SDS-PAGE and western blotting. Left lane; CTLL-2 conditioned media. Right lane; CHO-muOPG conditioned media. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 18. Detection of OPG expression in serum samples and liver extracts obtained from control and OPG transgenic mice. Transgenic mice were constructed as described in Example 4. OPG expression was visualized after SDS-PAGE followed by Western blotting using anti-OPG antibodies.

Figure 19. Effects of huOPG [22-401]-Fc fusion protein on osteoclast formation in vitro. The osteoclast forming assay was performed as described in Example 11A in the absence (control) or presence of the indicated amounts of huOPG [22-401]-Fc fusion. Osteoclast formation was visualized by histochemical staining for tartrate acid phosphatase (TRAP). A. OPG added to 100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to

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0.01 ng/ml. F. OPG added to 0.001 ng/ml. G. Control.
No OPG added.

- Figure 20. Decrease in osteoclast culture TRAP activity with increasing amounts of OPG. Indicated concentrations of huOPG [22-401]-Fc fusion protein were added to osteoclast forming assay and TRAP activity quantitated as described in Example 11A.
- 10 Figure 21. Effect of OPG on a terminal stage of osteoclast differentiation. huOPG [22-401]-Fc fusion was added to the osteoclast forming assay during the intermediate stage of osteoclast maturation (days 5-6; OPG-CTL) or during the terminal stage of osteoclast
- 15 maturation (days 7-15; CTL-OPG). TRAP activity was quantitated and compared with the activity observed in the absence of OPG (CTL-CTL) in the presence of OPG throughout (OPG-OPG).
- 20 Figure 22. Effects of IL-1 β , IL-1 α and OPG on blood ionized calcium in mice. Levels of blood ionized calcium were monitored after injection of IL-1 β alone, IL-1 α alone, IL-1 β plus muOPG [22-401]-Fc, IL-1 α plus
- 25 muOPG [22-401]-Fc, and muOPG [22-401]-Fc alone. Control mice received injections of phosphate buffered saline (PBS) only. IL-1 β experiment shown in A; IL-1 α experiment shown in B.
- 30 Figure 23. Effects of OPG on calvarial osteoclasts in control and IL1-treated mice. Histological methods for analyzing mice calvarial bone samples are described in Example 11B. Arrows indicate osteoclasts present in day 2-treated mice. Calvarial samples of mice receiving four PBS injections daily (A), one injection of IL-1 and

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three injections of PBS daily (B), one injection of PBS and three injections of OPG daily (C), one injection of IL-1 and three injections of OPG daily.

- 5 Figure 24. Radiographic analysis of bone accumulation in marrow cavity of normal mice. Mice were injected subcutaneously with saline (A) or muOPG [22-401]-Fc fusion (5mg/kg/d) for 14 days (B) and bone density determined as described in Example 11C.

10

Figure 25. Histomorphometric analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C.

15

Figure 26. Histology analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C.

- 20 A. Saline injection B. Injection of muOPG [22-401]-Fc fusion.

- Figure 27. Activity of OPG administered to ovariectomized rats. In this two week experiment the trend to reduced bone density appears to be blocked by OPG or other anti-resorptive therapies. DEXA measurements were taken at time of ovariectomy and at week 1 and week 2 of treatment. The results are expressed as % change from the initial bone density (Mean +/- SEM).

30

Figure 28. Bone density in the femoral metaphysis, measured by histomorphometric methods, tends to be lower in ovariectomized rats (OVX) than sham operated animals (SHAM) 17 days following ovariectomy. This effect was

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blocked by OPG-Fc, with OPG-Fc treated ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

5

Detailed Description of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an
10 expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library. The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are
15 shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of the full-length cDNA clones isolated encoded
20 transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human gene spanning nucleotides 1200-1353 shown in Figure 9D
25 was deposited in the Genbank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta
30 and heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and appendix.

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The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long bones (femurs), vertebrae and flat bones (pelvis).
5 Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and resorption which has led to a marked accumulation of bone and cartilage.
10 A decrease in the number of trabecular osteoclasts in the bones of OPG expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In view of the activity in
15 transgenic expressors, the TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is
20 described in Examples 7 and 8. Mouse OPG was produced as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion
25 polypeptides to the Fc region of human IgG1 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly
30 produced mammalian and bacterial OPG is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced

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hypercalcemia, and injection studies of bone density in normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in E. coli osteoclast

5 maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc, huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in E. coli did not demonstrate activity in the in vitro assay.

10 OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG [22-401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant polypeptide in CHO cells, and OPG expressed as a naturally occurring
15 product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS gels (see Example 9). Truncated OPG polypeptides having deletions in the region of amino acids 186-401 (e.g., OPG [1-185] and OPG [1-194]) were predominantly
20 monomeric suggesting that the region 186-401 may be involved in self-association of OPG polypeptides. However, huOPG met[32-401] produced in E. coli was largely monomeric.

OPG may be important in regulating bone
25 resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor-ligand interaction involved in the osteolytic pathway. One aspect of the regulation appears to be a reduction in the number of osteoclasts.

30

Nucleic Acids

The invention provides for an isolated nucleic acid encoding a polypeptide having at least one of the biological activities of OPG. As described herein, the

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biological activities of OPG include, but are not limited to, any activity involving bone metabolism and in particular, include increasing bone density. The nucleic acids of the invention are selected from the following:

- a) the nucleic acid sequences as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) or complementary strands thereof;
- b) the nucleic acids which hybridize under stringent conditions with the polypeptide-encoding region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124); and
- c) nucleic acids which hybridize under stringent conditions with nucleotides 148 through 337 inclusive as shown in Figure 1A.
- d) the nucleic acid sequences which are degenerate to the sequences in (a) and (b).

The invention provides for nucleic acids which encode rat, mouse and human OPG as well as nucleic acid sequences hybridizing thereto which encode a polypeptide having at least one of the biological activities of OPG. Also provided for are nucleic acids which hybridize to a rat OPG EST encompassing nucleotides 148-337 as shown in Figure 1A. The conditions for hybridization are generally of high stringency such as 5xSSC, 50% formamide and 42°C described in Example 1 of the specification. Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature. The nucleic acids in (b) encompass sequences encoding OPG-related polypeptides which do not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the nucleic acids are as shown in Figures 2B-2C (SEQ ID

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NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124).

5 The length of hybridizing nucleic acids of the invention may be variable since hybridization may occur in part or all of the polypeptide-encoding regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), and may also occur in adjacent noncoding regions. Therefore, hybridizing nucleic acids may be truncations or extensions of the sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated or extended nucleic acids are encompassed by the invention provided they retain one or more of the biological properties of OPG. The hybridizing nucleic acids may also include adjacent noncoding regions which are 5' and/or 3' to the OPG coding region. The noncoding regions include regulatory regions involved in OPG expression, such as promoters, enhance, translational initiation sites, transcription termination sites and the like.

Hybridization conditions for nucleic acids are described in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)

25 DNA encoding rat OPG was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under ATCC accession no. 69970. DNA encoding mouse OPG was provided in plasmid pRcCMV-murine OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under accession no. 69971. DNA encoding human OPG was provided in plasmid pRcCMV - human OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under

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accession no. 69969. The nucleic acids of the invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. 69969, 69970, and 69971 and have at least one of the biological activities of OPG.

Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2B, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution, insertion or deletion of one or more residues such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of OPG. The nucleic acid derivatives may be naturally occurring, such as by splice variation or polymorphism, or may be constructed using site-directed mutagenesis techniques available to the skilled worker. One example of a naturally occurring variant of OPG is a nucleic acid encoding a lys to asn change at residue 3 within the leader sequence (see Example 5). It is anticipated that nucleic acid derivatives will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a membrane-bound form of OPG having an extracellular domain as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with transmembrane and cytoplasmic domains.

In one embodiment, derivatives of OPG include nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the carboxy terminus. Nucleic acids encoding OPG may have from 1 to 216 amino acids deleted from the carboxy terminus. Optionally, an antibody Fc region may extend from the new carboxy

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terminus to yield a biologically active OPG-Fc fusion polypeptide. (see Example 11). In preferred embodiments, nucleic acids encode OPG having the amino acid sequence from residues 22-185, 22-189, 22-194 or 5 22-201 (using numbering in Figure 9E-F) and optionally, encoding an Fc region of human IgG.

Also included are nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the amino terminus. Truncated forms 10 include those lacking part or all the 21 amino acids comprising the leader sequence. Additionally, the invention provides for nucleic acids encoding OPG having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and ,optionally, having from 1 15 to 216 amino acids deleted from the carboxy terminus (at residue 401). Optionally, the nucleic acids may encode a methionine residue at the amino terminus. Examples of such OPG truncated polypeptides are described in Example 8.

20 Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing OPG. In humans, tissue sources for OPG include kidney, liver, placenta and 25 heart. Genomic DNA encoding OPG is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to 30 reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by procaryotic expression

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vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding OPG, host cells transformed with said vectors and methods for the production of OPG are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185, Goeddel, D.V. ed. Academic Press (1990).

Host cells for the production of OPG include procaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression. Preferred mammalian host cells include COS, CHOd-, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity. Mammalian expression allows for the production of

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secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of OPG contain at a minimum sequences required for vector propagation and for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of OPG are also included. Such vectors include promoters which function specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of OPG in targeted human cells.

Using an appropriate host-vector system, OPG is produced recombinantly by culturing a host cell transformed with an expression vector containing nucleic acid sequences encoding OPG under conditions such that OPG is produced, and isolating the product of expression. OPG is produced in the supernatant of transfected mammalian cells or in inclusion bodies of transformed bacterial host cells. OPG so produced may be purified by procedures known to one skilled in the art as described below. The expression of OPG in mammalian and bacterial host systems is described in Examples 7 and 8. Expression vectors for mammalian hosts are exemplified by plasmids such as pDSR α described in PCT Application No. 90/14363. Expression vectors for bacterial host cells are exemplified by plasmids pAMG21 and pAMG22-His described in Example 8. Plasmid pAMG21 was deposited with the American Type

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Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98113. Plasmid pAMG22-His was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98112. It is anticipated that the specific plasmids and host cells described are for illustrative purposes and that other available plasmids and host cells could also be used to express the polypeptides.

The invention also provides for expression of OPG from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of OPG from the host chromosome. Expression of OPG by the introduction of exogenous regulatory sequences (e.g. promoters or enhancers) capable of directing the production of OPG[™] from endogenous OPG coding regions is also encompassed. Stimulation of endogenous regulatory sequences capable of directing OPG production (e.g. by exposure to transcriptional enhancing factors) is also provided by the invention.

Polypeptides

The invention provides for OPG, a novel member of the TNF receptor superfamily, having an activity associated with bone metabolism and in particular having the activity of inhibiting bone resorption thereby increasing bone density. OPG refers to a polypeptide having an amino acid sequence of mouse, rat or human OPG or a derivative thereof having at least one of the biological activities of OPG. The amino acid sequences of rat, mouse and human OPG are shown in Figures 2B-2C (SEQ ID NO:121), 9A-9B (SEQ ID NO:123), and 9C-9D (SEQ ID NO:125) respectively. A derivative of OPG refers to a polypeptide having an addition, deletion, insertion or substitution of one or more amino acids such that the

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resulting polypeptide has at least one of the biological activities of OPG. The biological activities of OPG include, but are not limited to, activities involving bone metabolism. Preferably, the polypeptides will have the amino terminal leader sequence of 21 amino acids removed.

OPG polypeptides encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]-Fc fusion, rat [1-180]-Fc fusion, mouse [1-401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180], human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in particular deletion of the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids deleted from the carboxy terminus. In another embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and, optionally, has from 1 to about 216 amino acids deleted from the carboxy terminus.

Additional OPG polypeptides encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian

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host cells, such as CHO or 293 cells, Additional OPG polypeptides encompassed by the invention which are expressed in procaryotic host cells include the following: human met[22-401], Fc-human met[22-401] fusion (Fc region is fused at the amino terminus of the full-length OPG coding sequence as described in Example 8), human met[22-401]-Fc fusion (Fc region fused to the full-length OPG sequence), Fc-mouse met[22-401] fusion, mouse met[22-401]-Fc fusion, human met[27-401], human met[22-185], human met[22-189], human met[22-194], human met[22-194] (P25A), human met [22-194] (P26A), human met[27-185], human met[27-189], human met[27-194], human met-arg-gly-ser-(his)₆ [22-401], human met-lys [22-401], human met-(lys)₃-[22-401], human met[22-401]-Fc (P25A), human met[22-401] (P25A), human met[22-401] (P26A), human met[22-401] (P26D), mouse met[22-401], mouse met[27-401], mouse met[32-401], mouse met[27-180], mouse met[22-189], mouse met[22-194], mouse met[27-189], mouse met[27-194], mouse met-lys[22-401], mouse HEK[22-401] (A45T), mouse met-lys-(his)₇[22-401], mouse met-lys[22-401]-(his)₇ and mouse met[27-401] (P33E, G36S, A45P). It is understood that the above OPG polypeptides produced in procaryotic host cells have an amino-terminal methionine residue, if such a residue is not indicated. In specific examples, OPG-Fc fusion were produced using a 227 amino acid region of human IgG1- γ 1 was used having the sequence as shown in Ellison et al. (Nuc. Acids Res. 10, 4071-4079 (1982)). However, variants of the Fc region of human IgG may also be used.

Analysis of the biological activity of carboxy-terminal OPG truncations fused to the human IgG1 Fc region indicates a portion of OPG of about 164 amino acids which is required for activity. This region encompasses amino acids 22-185, preferably those in

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Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extraceullular domains.

Using the homology between OPG and the extracellular ligand binding domains of TNF receptor family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG which may be important for biological activity. Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 to cys62, tyr23 and his 66 may act to stabilize the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which were in close proximity to TNF β as shown in Figures 11 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNF β was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following residues in OPG may be important for ligand binding: glu34, lys43, pro66 to gln91 (in particular, pro66, his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual cysteine-rich domains, whereas changes in

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residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable properties, such as enhanced biological activity, greater stability, or greater ease of formulation.

The invention also provides for an OPG multimer comprising OPG monomers. OPG appears to be active as a multimer (e.g., dimer, trimer of a higher number of monomers). Preferably, OPG multimers are dimers or trimers. OPG multimers may comprise monomers having the amino acid sequence of OPG sufficient to promote multimer formation or may comprise monomers having heterologous sequences such as an antibody Fc region. Analysis of carboxy-terminal deletions of OPG suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of self-association is also encompassed by the invention. Alternatively, OPG polypeptides or derivatives thereof may be modified to form dimers or multimers by site directed mutagenesis to create unpaired cysteine residues for interchain disulfide bond formation, by photochemical crosslinking, such as exposure to ultraviolet light, or by chemical crosslinking with bifunctional linker molecules such as bifunctional polyethylene glycol and the like.

Modifications of OPG polypeptides are encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked

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or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for
5 detection and isolation of the protein.

Further modifications of OPG include chimeric proteins wherein OPG is fused to a heterologous amino acid sequence. The heterologous sequence may be any
10 sequence which allows the resulting fusion protein to retain the activity of OPG. The heterologous sequences include for example, immunoglobulin fusions, such as Fc fusions, which may aid in purification of the protein. A heterologous sequence which promotes association of
15 OPG monomers to form dimers, trimers and other higher multimeric forms is preferred.

The polypeptides of the invention are isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing OPG, or
20 purified from components in cell cultures containing the secreted protein. In one embodiment, the polypeptide is free from association with other human proteins, such as the expression product of a bacterial host cell.

Also provided by the invention are chemically
25 modified derivatives of OPG which may provide additional advantages such as increasing stability and circulating time of the polypeptide, or decreasing immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble
30 polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined

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positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g. EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group

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for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

5 One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion
10 of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation
15 (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemically modification may be accomplished by reductive
20 alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of
25 the protein at the N-terminus with a carbonyl group containing polymer is achieved.

Synthetic OPG dimers may be prepared by various chemical crosslinking procedures. OPG monomers may be chemically linked in any fashion that retains or
30 enhances the biological activity of OPG. A variety of chemical crosslinkers may be used depending upon which properties of the protein dimer are desired. For example, crosslinkers may be short and relatively rigid or longer and more flexible, may be biologically

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reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see Example 12). In the first step, OPG is chemically modified at the amino terminus to introduce a protected thiol, which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG molecule. Amino-terminal crosslinks include, but are not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG "dumbbells"). Also encompassed by PEG dumbbell synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG may be crosslinked directly through a variety of amine specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS³) as well as others known in the art. It is also possible to thiolate OPG directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.

A method for the purification of OPG from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The

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chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity complex and the like.

Antibodies

Also encompassed by the invention are antibodies specifically binding to OPG. Antigens for the generation of antibodies may be full-length polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.

Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CnBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to

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remove OPG from liquid samples. Antibodies are also used as diagnostic reagents to detect and quantitate OPG in biological samples by methods described below.

5 Pharmaceutical compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the polypeptide of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascorbic acid or sodium metabisulfite. Also encompassed are compositions comprising OPG modified with water soluble polymers to increase solubility or stability. Compositions may also comprise incorporation of OPG into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Specifically, OPG compositions may comprise incorporation into polymer matrices such as hydrogels, silicones, polyethylenes, ethylene-vinyl acetate copolymers, or biodegradable polymers. Examples of hydrogels include polyhydroxyalkylmethacrylates (p-HEMA), polyacrylamide, polymethacrylamide, polyvinylpyrrolidone, polyvinyl alcohol and various polyelectrolyte complexes. Examples of biodegradable polymers include polylactic acid (PLA), polyglycolic

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acid (PGA), copolymers of PLA and PGA, polyamides and copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric beads which may be administered by injection.

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the OPG coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

30 Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes,

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osteoblasts and osteoclasts, are involved in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption.

5 Resorption, or the dissolution of bone matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in
10 resorption induced by hormones. For instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of
15 calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone
20 formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts
25 earlier in females than males and is distinctly accelerated after menopause in some females (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels.
30 Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of

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osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder characterized by a net bone loss (osteopenia or osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

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Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

Paget's disease of bone (osteitis deformans) in adults and juveniles

Osteomyelitis, or an infectious lesion in bone, leading to bone loss.

Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia), idiopathic hypercalcemia, and hypercalcemia associated with hyperthyroidism and renal function disorders.

Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases.

Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions.

Bone loss due to rheumatoid arthritis.

Periodontal bone loss.

Osteolytic metastasis

It is understood that OPG may be used alone or in conjunction with other factors for the treatment of bone disorders. In one embodiment, osteoprotegerin is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12,

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transforming growth factor- β (TGF- β) and TGF- β family members, interleukin-1 inhibitors, TNF α inhibitors, parathyroid hormone and analogs thereof, parathyroid related protein and analogs thereof, E series
5 prostaglandins, bisphosphonates (such as alendronate and others), and bone-enhancing minerals such as fluoride and calcium.

The following examples are offered to more
10 fully illustrate the invention, but are not construed as limiting the scope thereof.

15

EXAMPLE 1

Identification and isolation of the rat OPG cDNA

Materials and methods for cDNA cloning and
20 analysis are described in Maniatis et al, *ibid*. Polymerase chain reactions (PCR) were performed using a Perkin-Elmer 9600 thermocycler using PCR reaction mixture (Boehringer-Mannheim) and primer concentrations specified by the manufacturer. In general, 25-50 μ l
25 reactions were denatured at 94°C, followed by 20-40 cycles of 94°C for 5 seconds, 50-60°C for 5 seconds, and 72°C for 3-5 minutes. Reactions were then treated for 72 °C for 3-5 minutes. Reactions were then analyzed by gel electrophoresis as described in Maniatis et al., *ibid*.

30

A cDNA library was constructed using mRNA isolated from embryonic d20 intestine for EST analysis (Adams et al. Science 252, 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total

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cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA

5 preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A random primed cDNA library was prepared using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md). The random cDNA
10 primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5'-AAAGGAAGGAAAAAAGCGGCCGCTACANNNNNNNT-3' (SEQ ID NO:1)

Not I

15 For the first strand synthesis three separate reactions were assembled that contained 2.5 µg of poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random primer. After second strand synthesis, the reaction products were separately extracted with a mixture of
20 phenol:chloroform:isoamyl alcohol (25:24:1 ratio), and then ethanol precipitated. The double strand (ds) cDNA products of the three reactions were combined and ligated to the following ds oligonucleotide adapter:

25 5'-TCGACCCACGCGTCCG-3' (SEQ ID NO:2)

3'-GGGTGCGCAGGCp-5' (SEQ ID NO:3)

After ligation the cDNA was digested to completion with Not I, extracted with
30 phenol:chloroform:isoamyl (25:24:1) alcohol and ethanol precipitated. The resuspended cDNA was then size fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer.

- 40 -

The two fractions containing the largest cDNA products were pooled, ethanol precipitated and then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann et al, 1991). The ligated cDNA was introduced into competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented LB nutrient media. The colonies that arose were picked and arrayed onto 96 well microtiter plates containing 200 µl of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin replicating tool, then both sets were stored at -80°C for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was separately isolated using the Qiagen Plasmid Maxi Kit (Qiagen Corp., Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots diluted 1:25 in distilled. Approximately 3.0 µl of diluted bacterial cultures were added to PCR reaction mixture (Boehringer-Mannheim) containing the following oligonucleotides:

5'-TGTAACGACGGCCAGT-3' (SEQ ID NO:4)
5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions:

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94 C for 2 minutes; 30 cycles of 94°C for 5 seconds, 50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4 minutes. After incubation in the thermocycler, the reactions were diluted with 2.0 mL of water. The amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on an Applied Biosystems 373A automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5'-CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq dye-terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures.

The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson et al. Meth. Enzymol. 183, (1990)). Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known members of the tumor necrosis factor receptor (TNFR) superfamily (Smith et al. Cell 76, 959-962 (1994)), using the sequence profile method of Gribskov et al. (Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as modified by Luethy et al. (Protein Science 3, 139-146 (1994)).

Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a possible new member of the TNFR superfamily. FRI-1 contained an approximately 600 bp insert with a LORF of about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-2). The region compared showed an ~43% homology between TNFR-2 and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR

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superfamily yielded a Z score of ~8, indicating that the FRI-1 gene possibly encodes a new family member. To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length clones. The following oligonucleotides were derived from the original FRI-1 sequence:

5'-GCATTATGACCCAGAAACCGGAC-3' (SEQ ID NO:7)

5'-AGGTAGCGCCCTTCCTCACATTC-3' (SEQ ID NO:8)

10

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in a PCR reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cycler with the following cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at 94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel electrophoresis. 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform competent ElectroMAX DH10B *E. coli* (Gibco BRL, Gaithersburg, MD) as described above. Approximately 40,000 transformants were plated onto sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a ³²p-dCTP labelled version of the PCR product obtained above. Filters were prehybridized in 5X SSC, 50% deionized formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C. Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml

25
30

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denatured salmon sperm DNA, and ~5 ng/ml of labelled probe for ~18 hours at 42°C. The filters were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C.

- 5 Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml
10 ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA search of the public database to detect any existing sequence matches and/or similarities. No matches to any
15 known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-2 genes. A methionine start codon is found at bp 124 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa
20 residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program
25 (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches, although there appeared to be a strong similarity to many members of the TNFR superfamily, most notably the
30 human and mouse TNFR-2. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and then modified by PrettyPlot (Wisconsin GCG package, version 8.1). This alignment shows a clear homology between the full

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length FRI-1 gene product and all other TNFR family members. The homologous region maps to the extracellular domain of TNFR family members, and corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted receptor, similar to TNFR-1 derived soluble receptors (Kohn et al. Proc. Natl. Acad. Sci. USA 87, 8331-8335 (1990)). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin (OPG).

15

EXAMPLE 2

OPG mRNA Expression Patterns in Tissues

Multiple human tissue northern blots (Clontech) were probed with a ³²P-dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to determine patterns of expression. Northern blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

30

Using a probe derived from the rat gene, a predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest

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levels are detected in the kidney. A large mRNA species of Mr 4.5 and 7.5 kb was detected in skeletal muscle and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA.

5 Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In addition, relatively high levels of the 2.4 kb transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript

10 detected by both the rat and human Osteoprotegerin gene is almost identical to the length of the rat pB1.1 FRI-1 insert, suggesting it was a full length cDNA clone.

EXAMPLE 3

15 Systemic delivery of OPG in transgenic mice

The rat OPG clone pB1.1 was used as template to PCR amplify the coding region for subcloning into an ApoE-liver specific expression vector (Simonet et al. J. Clin. Invest. 94, 1310-1319 (1994), and PCT Application

20 No. US94/11675 and co-owned U.S. Serial No. 08/221,767. The following 5' and 3' oligonucleotide primers were used for PCR amplification, respectively:

25 5'-GACTAGTCCCACAATGAACAAGTGGCTGTG-3' (SEQ ID NO:9)
5'-ATAAGAATGCGGCCGCTAACTATGAAACAGCCCAGTGACCATTG-3'
(SEQ ID NO:10)

The PCR reaction mixture (Boehringer-Mannheim)

30 was treated as follows: 94°C for 1 minute, 1 cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1 minute, 25 cycles. Following amplification, the samples were purified over Qiagen PCR columns and digested overnight with SpeI and NotI restriction enzymes. The digested

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products were extracted and precipitated and subcloned into the ApoE promoter expression vector. Prior to microinjecting the resulting clone, HE-OPG, it was sequenced to ensure it was mutation-free.

5 The HE-OPG plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid DNA was digested with XhoI and Ase I, and the 3.6 kb transgene insert was purified by gel electrophoresis. The purified fragment was diluted to a
10 stock injection solution of 1 µg/ml in 5 mM Tris, pH 7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x BDF1-bred mice were injected essentially as described (Brinster et al., Proc. Natl. Acad. Sci. USA 82, 4338 (1985)), except that injection needles were beveled and
15 siliconized before use. Embryos were cultured overnight in a CO₂ incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

 Following term pregnancy, 49 offspring were
20 obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the human Apo E intron which was included in the expression
25 vector. The oligos used for PCR amplification were:

5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)

5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

30 The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were identified as PCR positive transgenic founders.

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At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls (1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the remaining 4 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as described (McDonald et al. Meth. Enzymol. 152, 219 (1987)). Northern blot analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden et al. Meth. Enzymol. 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with ³²P dCTP-labelled pB1.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and 2-4 x 10⁶ cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2, 11, 16, 17, 22, 33, and 45). The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since OPG is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and

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northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

5

EXAMPLE 4

Biological activity of OPG

10 Five of the transgenic mice (animals
2,11,16,17 and 28) and 5 control littermates (animals
1,12,15,18, and 30) were sacrificed for necropsy and
pathological analysis using the following procedures:
Prior to euthanasia, all animals had their
15 identification numbers verified, then were weighed,
anesthetized and blood drawn. The blood was saved as
both serum and whole blood for a complete serum
chemistry and hematology panel. Radiography was
performed just after terminal anesthesia by lethal CO₂
20 inhalation, and prior to the gross dissection.
Following this, tissues were removed and fixed in 10%
buffered Zn-Formalin for histological examination. The
tissues collected included the liver, spleen, pancreas,
stomach, duodenum, ileum, colon, kidney, reproductive
25 organs, skin and mammary glands, bone, brain, heart,
lung, thymus, trachea, esophagus, thyroid, jejunum,
cecum, rectum, adrenals, urinary bladder, and skeletal
muscle. Prior to fixation the whole organ weights were
determined for the liver, stomach, kidney, adrenals,
30 spleen, and thymus. After fixation the tissues were
processed into paraffin blocks, and 3 um sections were
obtained. Bone tissue was decalcified using a formic
acid solution, and all sections were stained with
hematoxylin and eosin. In addition, staining with

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Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate resistant acid phosphatase (TRAP), an enzyme highly expressed by
5 osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage. Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect replicating cells and cells of the monocyte-macrophage lineage, respectively. To
10 detect F480 surface antigen expression, formalin fixed, paraffin embedded 4 μ m sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat
15 monoclonal anti-mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated streptavidin (BioGenex San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with
20 hematoxylin.

Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by
25 approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In
30 addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The

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relative size of femurs in the expressors were not different from the the control mice.

Histological analysis of stained sections of bone from the OPG expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the OPG-induced skeletal changes were systemic. The residual bone marrow showed predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-macrophage derivation in the mouse, showed the presence of F480 positive cells in the marrow spaces. Focally, flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

The mesenchymal cells lining the bony trabeculae were flattened and appeared inactive. Based on H&E and TRAP stains, osteoclasts were rarely found on the trabecular bone surfaces in the OPG expressors. In contrast, osteoclasts and/or chondroclasts were seen in the region of the growth plate resorbing cartilage, but their numbers may be reduced compared to controls. Also, osteoclasts were present on the cortical surface of the metaphysis where modelling activity is usually robust. The predominant difference between the expressors and controls was the profound decrease in trabecular osteoclasts, both in the vertebrae and femurs. The extent of bone accumulation was directly correlated with

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the level of OPG transgene mRNA detected by northern blotting of total liver RNA.

The spleens from the OPG expressors had an increased amount of red pulp with the expansion due to increased hematopoiesis. All hematopoietic lineages are represented. F480 positive cells were present in both control and OPG expressors in the red pulp. Two of the expressors (2 and 17) had foci of extramedullary hematopoiesis within the liver and this is likely due to the osteopetrotic marrow.

There were no observable abnormalities in the thymus, lymph nodes, gastrointestinal tract, pancreato-hepatobiliary tract, respiratory tract, reproductive system, genito-urinary system, skin, nervous system, heart and aorta, breast, skeletal muscle and fat.

EXAMPLE 5

Isolation of mouse and human OPG cDNA

A cDNA clone corresponding to the 5' end of the mouse OPG mRNA was isolated from a mouse kidney cDNA library (Clontech) by PCR amplification. The oligonucleotides were derived from the rat OPG cDNA sequence and are shown below:

5'-ATCAAAGGCAGGGCATACTTCCTG-3' (SEQ ID NO:13)

5'-GTTGCACTCCTGTTTCACGGTCTG-3' (SEQ ID NO:14)

5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)

5'-TAACTTTTACAGAAGAGCATCAGC-3' (SEQ ID NO:16)

5'-AGCGCGGCCGCATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)

5'-AGCTCTAGAGAAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)

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The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and Xba I, then directionally cloned into the plasmid vector pRcCMV (Invitrogen). The resulting plasmid was named pRcCMV-Mu-OPG. The nucleotide sequence of the cloned product was compared to the rat OPG cDNA sequence. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 88% identical. The mouse cDNA sequence contained a 401 aa LORF, which was compared to the rat OPG protein sequence and found to be ~94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine OPG protein, and that the sequence and structure has been highly conserved throughout evolution. The mouse OPG protein sequence contains an identical putative signal peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

A partial human OPG cDNA was cloned from a human kidney cDNA library using the following rat-specific oligonucleotides:

5'-GTG AAG CTG TGC AAG AAC CTG ATG-3'
(SEQ ID NO:19)
5'-ATC AAA GGC AGG GCA TAC TTC CTG-3'
(SEQ ID NO:20)

This PCR product was sequenced and used to design primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:

5'-TCCGTAAGAAACAGCCCAAGTGACC-3' (SEQ ID NO:29)

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5'-CAGATCCTGAAGCTGCTCAGTTTG-3' (SEQ ID NO:21)

5 The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design 5' and 3' human-specific primers useful for amplifying the entire human OPG cDNA coding sequences:

5'-AGCGCGGCCGCGGGGACCACAATGAACAAGTTG-3' (SEQ ID NO:22)

5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)

10

The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human OPG. The nucleotide sequence of the cloned product was compared to the rat and mouse OPG cDNA sequences. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 78-88% identical to the human OPG cDNA. The human OPG cDNA sequence also contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human OPG protein is approximately 85% identical, and ~90% identical to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked glycosylation, 4 of which are conserved between the rat and mouse OPG proteins.

30 The DNA and predicted amino acid sequence of mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122). The DNA and predicted amino acid sequence of human OPG is shown in Figure 9C and 9D (SEQ ID NO:124). A

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comparison of the rat, mouse and human OPG amino acid sequences is shown in Figure 9E and 9F.

Isolation of additional human OPG cDNA clones revealed the presence of a G to C base change at
5 position 103 of the DNA sequence shown in Figure 9C. This nucleotide change results in substitution of an asparagine for a lysine at position 3 of the amino acid sequence shown in Figure 9C. The remainder of the
10 sequence in clones having this change was identical to that in Figure 9C and 9D.

EXAMPLE 6

OPG three-dimensional structure modelling

15

The amino-terminal portion of OPG has homology to the extracellular portion of all known members of the TNFR superfamily (Figure 1C). The most notable motif in this region of TNFR-related genes is an ~40 amino acid,
20 cysteine-rich repeat sequence which folds into distinct structures (Banner et al. Cell 73, 431-445 (1993)). This motif is usually displayed in four (range 3-6) tandem repeats (see Figure 1C), and is known to be involved in ligand binding (Beutler and van Huffer
25 Science 264, 667-663 (1994)). Each repeat usually contains six interspaced cysteine residues, which are involved in forming three intradomain disulfide bonds, termed SS1, SS2, and SS3 (Banner et al., *ibid*). In some
30 receptors, such as TNFR2, CD30 and CD40, some of the repeat domains contain only two intrachain disulfide bonds (SS1 and SS3).

The human OPG protein sequence was aligned to a TNFR1 extracellular domain profile using methods described by Luethy, et al., *ibid*, and the results were

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graphically displayed using the PrettyPlot program from the Wisconsin Package, version 8.1 (Genetics Computer Group, Madison, WI) (Figure 10). The alignment indicates a clear conservation of cysteine residues involved in formation of domains 1-4. This alignment was then used to construct a three-dimensional (3-D) model of the human OPG N-terminal domain using the known 3-D structure of the extracellular domain of p55 TNFR1 (Banner et al., *ibid*) as the template. To do this the atomic coordinates of the peptide backbone and side chains of identical residues were copied from the crystal structure coordinates of TNFR1. Following this, the remaining coordinates for the insertions and different side chains were generated using the LOOK program (Molecular Applications Group, Palo Alto, CA). The 3-D model was then refined by minimizing its conformational energy using LOOK.

By analogy with other TNFR family members, it is assumed that OPG binds to a ligand. For the purpose of modelling the interaction of OPG with its ligand, the crystal structure of TNF- β was used to simulate a 3-D representation of an "OPG ligand". This data was graphically displayed (see Figure 11) using Molscript (Kraulis, J. Appl. Cryst. 24, 946-950, 1991). A model for the OPG/ligand complex with 3 TNF β and 3 OPG molecules was constructed where the relative positions of OPG are identical to TNFR1 in the crystal structure. This model was then used to find the residues of OPG that could interact with its ligand using the following approach: The solvent accessible area of all residues in the complex and one single OPG model were calculated. The residues that have different accessibility in the complex than in the monomer are likely to interact with the ligand.

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The human and mouse OPG amino acid sequences were realigned using this information to highlight sequences comprising each of the cysteine rich domains 1-4 (Figure 12A and 12B). Each domain has individual structural characteristics which can be predicted:

Domain 1

Contains 4 cysteines involved in SS2 (C41 to C54) and SS3 (C44 to C62) disulfide bonds. Although no SS1 bond is evident based on disulfide bridges, the conserved tyrosine at position 28 is homologous to Y20 in TNFR1, which is known to be involved in interacting with H66 to aid in domain formation. OPG has a homologous histidine at position 75, suggesting OPG Y28 and H75 stack together in the native protein, as do the homologous residues in TNFR1. Therefore, both of these residues may indeed be important for biological activity, and N-terminal OPG truncations up to and beyond Y28 may have altered activity. In addition, residues E34 and K43 are predicted to interact with a bound ligand based on our 3-dimensional model.

Domain 2

Contains six cysteines and is predicted to contain SS1 (C65 to C80), SS2 (C83 to C98) and SS3 (C87 to C105) disulfide bonds. This region of OPG also contains an region stretching from P66-Q91 which aligns to the portion of TNFR1 domain 2 which forms close contacts with TNF β (see above), and may interact with an OPG ligand. In particular residues P66, H68, Y69, Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82, P85, V86, K88, E89, L90, and Q91 are predicted to interact with a bound ligand based on our structural data.

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Domain 3

Contains 4 cysteines involved in SS1 (C107 to C 118) and SS3 (C124 to C142) disulfide bonds, but not an SS2 bond. Based on our structural data, residues
5 E115, L118 and K119 are predicted in to interact with an OPG ligand.

Domain 4

Contains 4 cysteines involved in SS1 (C145 to C160) and SS3 (C166 to C185) disulfide bonds, but not an
10 SS2 bond, similar to domain 3. Our structural data predict that E153 and S155 interact with an OPG ligand.

Thus, the predicted structural model for OPG
15 identifies a number of highly conserved residues which are likely to be important for its biological activity.

EXAMPLE 7

20 Production of recombinant secreted OPG protein in mammalian cells

To determine if OPG is actually a secreted protein, mouse OPG cDNA was fused to the human IgG1 Fc domain as a tag (Capon et al. Nature 337, 525-531
25 (1989)), and expressed in human 293 fibroblasts. Fc fusions were carried out using the vector pFc-A3. pFc-A3 contains the region encoding the Fc portion of human immunoglobulin IgG- γ 1 heavy chain (Ellison et al. ibid)
30 from the first amino acid of the hinge domain (Glu-99) to the carboxyl terminus and is flanked by a 5'-NotI fusion site and 3'-SalI and XbaI sites. The plasmid was constructed by PCR amplification of the human spleen cDNA library (Clontech). PCR reactions were in a final

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volume of 100 μ l and employed 2 units of Vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 μ M (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100 with 400 μ M each dNTP and 1 ng of the cDNA library to be amplified together with 1 μ M of each primer. Reactions were initiated by denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 73°C for 2 min. The 5' primer

5' ATAGCGGCCGCTGAGCCCAAATCTTGTGACAAACTCAC 3' (SEQ ID NO:24)

incorporated a NotI site immediately 5' to the first residue (Glu-99) of the hinge domain of IgG- γ 1. The 3' primer

5'-TCTAGAGTCGACTTATCATTTACCCGGAGACAGGGAGAGGCTCTT-3' (SEQ ID NO:25)

incorporated SalI and XbaI sites. The 717-bp PCR product was digested with NotI and SalI, isolated by electrophoresis through 1% agarose (FMC Corp.), purified by the GeneClean procedure (BIO 101, Inc.) and cloned into NotI, SalI-digested pBluescript II KS vector (Stratagene). The insert in the resulting plasmid, pFc-A3, was sequenced to confirm the fidelity of the PCR reaction.

The cloned mouse cDNA in plasmid pRCCMV-MuOPG was amplified using the following two sets of primer pairs:

Pair 1

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:26)

5'-CCTCTGCGGCCGCTAAGCAGCTTATTTTACGGATTGAACCTG-3' (SEQ ID NO:27)

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Pair 2

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:28)

5'-CCTCTGCGGCCGCTGTTGCATTTCTTTCTG-3' (SEQ ID NO:30)

5

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction site 5' to aspartic acid residue 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the OPG protein and the IgG Fc region. This product, when linked to the Fc portion, would encode all 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc). The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 Fc domain (CT.fc).

The amino acid sequence junction linking OPG residue 401 and aspartic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted as described below, or the cysteine residue corresponding to C220 of the human Fc region can be mutated to either serine or alanine. OPF-Fc fusion protein encoded by these modified vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

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Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells.

5 The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 µg/ml hygromycin to select for vector expression, and the resulting drug-resistant mass cultures were grown to confluence. The cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced by the drug resistant 293 cultures. In the pCEP4-F1.Fc and the pCEP4-CT.Fc conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it may be stable. Both Fc fusion proteins were detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira et al. (J. Biol. Chem. 262, 10-35 (1987)). The following amino acid sequence was read after 19 cycles:

NH₂-E T L P P K Y L H Y D P E T G H Q L L-CO₂H
(SEQ ID NO:31)

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This sequence was identical to the predicted mouse OPG amino acid sequence beginning at amino acid residue 22, suggesting that the natural mammalian leader cleavage site is between amino acid residues Q21-E22, not between Y31-D32 as originally predicted. The expression experiments performed in 293-EBNA cells with pCEP4-F1.Fc and pCEP4-CT.Fc demonstrate that OPG is a secreted protein, and may act systemically to bind its ligand.

Procedures similar to those used to construct and express the muOPG[22-180]-Fc and muOPG[22-401]-Fc fusions were employed for additional mouse and human OPG-Fc fusion proteins.

Murine OPG cDNA encoding amino acids 1-185 fused to the Fc region of human IgG1 [muOPG Ct(185).Fc] was constructed as follows. Murine OPG cDNA from plasmid pRcCMV Mu Osteoprotegerin (described in Example 5) was amplified using the following primer pair in a polymerase chain reaction as described above:

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:32)

1333-80:

5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-3'

(SEQ ID NO:33)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 63-185 (corresponding to bp 278-645) of the OPG reading frame as shown in Figure 9A. The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFCA3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was purified, cleaved

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with NotI and EcoRI, and the resulting EcoRI-NotI restriction fragment was purified. The vector pCEP4 having the murine 1-401 OPG-Fc fusion insert was cleaved with EcoRI and NotI, purified, and ligated to the PCR
5 product generated above. The resulting pCEP4-based expression vector encodes OPG residues 1-185 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-185.Fc fusion vector was transfected into 293 cells, drug selected, and
10 conditioned media was produced as described above. The resulting secreted murine OPG 1-185.Fc fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

15 Murine OPG DNA encoding amino acid residues 1-194 fused to the Fc region of human IgG1 (muOPG Ct(194).Fc) was constructed as follows. Mouse OPG cDNA from plasmid pRcCMV Mu-Osteoprotegerin was amplified using the following primer pairs:

20

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:34)

1333-81:

25 5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-3'
(SEQ ID NO:35)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 70-194 (corresponding to bp 298-672) of the OPG reading frame.
30 The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFcA3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was cloned into the murine OPG[1-401] Fc fusion

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vector as described above. The resulting pCEP4-based expression vector encodes OPG residues 1-194 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-194.Fc fusion vector
5 was transfected into 293 cells, drug selected, and conditioned media was produced. The resulting secreted fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

10

Human OPG DNA encoding amino acids 1-401 fused to the Fc region of human IgG1 was constructed as follows. Human OPG DNA in plasmid pRcCMV-hu osteoprotegerin (described in Example 5) was amplified,
15 using the following oligonucleotide primers:

1254-90:

5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'
(SEQ ID NO:36)

20

1254-95:

5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'
(SEQ ID NO:37)

25

The resulting PCR product encodes the full-length human OPG protein and creates a Not I restriction site which is compatible with the in-frame Not I site Fc fusion vector FcA3. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above.
30 The resulting expression vector encodes human OPG residues 1-401 directly followed by 227 amino acid residues of the human IgG1 Fc region. Conditioned media from transfected and drug selected cells was produced and the huOPG F1.Fc fusion product was purified by

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Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-
5 201 fused to the Fc region of human IgG1 [huOPG
Ct(201).Fc] was constructed as follows. The cloned human
OPG cDNA from plasmid pRrCMV-hu osteoprotegerin was
amplified by PCR using the following oligonucleotide
primer pair:

10

1254-90:

5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT
G-3' (SEQ ID NO:38)

1254-92:

15

5'-CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'
(SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA
region encoding amino acid residues 1-201 of the OPG
20 reading frame, and creates a Not I restriction site at
the 3' end which is compatible with the in-frame Not I
site Fc fusion vector FcA3. This product, when linked
to the Fc portion, encodes OPG residues 1-201 directly
followed by all 221 amino acid residues of the human
25 IgG1 Fc region. The PCR product was directionally
cloned into the plasmid vector pCEP4 as described above.
Conditioned media from transfected and drug selected
cells was produced, and the hu OPG Ct(201).Fc fusion
products purified by Protein-A column chromatography
30 (Pierce) using the manufacturer's recommended
procedures.

The following procedures were used to
construct and express unfused mouse and human OPG.

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A plasmid for mammalian expression of full-length murine OPG (residues 1-401) was generated by PCR amplification of the murine OPG cDNA insert from pRcCMV Mu-Osteoprotegerin and subcloned into the expression vector pDSR α (DeClerck et. al. J. Biol. Chem. 266, 3893 (1991)). The following oligonucleotide primers were used:

1295-26:

10 5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT
GC-3' (SEQ ID NO:40)

1295-27:

15 5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG
ATT G-3' (SEQ ID NO:41)

The murine OPG full length reading frame was amplified by PCR as described above. The PCR product was purified and digested with restriction endonucleases Hind III and Xba I (Boehringer Mannheim, Indianapolis, IN) under the manufacturers recommended conditions, then ligated to Hind III and Xba I digested pDSR α . Recombinant clones were detected by restriction endonuclease digestion, then sequenced to ensure no mutations were produced during the PCR amplification steps.

The resulting plasmid, pDSR α -muOPG was introduced into Chinese hamster ovary (CHO) cells by calcium mediated transfection (Wigler et al. Cell 11, 233 (1977)). Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the murine OPG recombinant protein was monitored by western blot analysis of CHO

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cell conditioned media. High expressing cells were selected, and OPG expression was further amplified by treatment with methotrexate as described (DeClerck et al., *ibid*). Conditioned media from CHO cell lines was
5 produced for further purification of recombinant secreted murine OPG protein.

A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by
10 subcloning the cDNA insert in pRCCMV-hu Osteoprotegerin directly into vector pDSR α (DeClerck et al., *ibid*). The pRCCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion with Xba I. Vector DNA was digested with Hind III,
15 blunt ended with Klenow, then digested with Xba I, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

The resulting plasmid pDSR α -huOPG was
20 introduced into Chinese hamster ovary (CHO) cells as described above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the human OPG recombinant
25 protein was monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from CHO cell lines expressing human OPG was produced for protein
30 purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG

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cDNA from pRcCMV-Mu OPG was amplified using the following oligonucleotide primers:

1333-82:

5 5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42)

1356-12:

5'-CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT
GC-3' (SEQ ID NO:43)

10 This primer pair amplifies the murine OPG cDNA
region encoding amino acids 63-185 of the OPG reading
frame (bp 278-645) and contains an artificial stop codon
directly after the cysteine codon (C185), which is
15 followed by an artificial Sal I restriction endonuclease
site. The predicted product contains an internal Eco RI
restriction site useful for subcloning into a pre-
existing vector. After PCR amplification, the resulting
purified product was cleaved with Eco RI and Sal I
20 restriction endonucleases, and the large fragment was
gel purified. The purified product was then subcloned
into the large restriction fragment of an Eco RI and Sal
I digest of pBluescript-muOPG Fl.Fc described above.
The resulting plasmid was digested with Hind III and Xho
I and the small fragment was gel purified. This
25 fragment, which contains a open reading frame encoding
residues 1-185 was then subcloned into a Hind III and
Xho I digest of the expression vector pCEP4. The
resulting vector, pmuOPG [1-185], encodes a truncated
OPG polypeptide which terminates at a cysteine residue
30 located at position 185. Conditioned media from
transfected and drug selected cells was produced as
described above.

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1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:

5 5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG
TT-3' (SEQ ID NO:45)

This primer pair amplifies the murine OPG cDNA region encoding amino acids 70-194 of the OPG reading frame (bp 298-672) and contains an artificial stop codon directly after the lysine codon (K194), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG Fl.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains a open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a lysine at position 194. Conditioned media from transfected and drug selected cells was produced as described above.

Several mutations were generated at the 5' end of the huOPG [22-401]-Fc gene that introduce either amino acid substitutions, or deletions, of OPG between residues 22 through 32. All mutations were generated

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with the "QuickChange™ Site-Directed Mutagenesis Kit" (Stratagene, San Diego, CA) using the manufacturer's recommended conditions. Briefly, reaction mix containing huOPG [22-401]-Fc plasmid DNA template and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent *E. coli* XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to delete residues 22-26 of the human OPG gene, resulting in the production of a huOPG [27-401]-Fc fusion protein:

1436-11:

5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3'
(SEQ ID NO:140)

1436-12:

5'-GTC ATA ATG AAG GTA CTT CTG GGT GGT CCA-3'
(SEQ ID NO:141)

The following primer pairs were used to delete residues 22-28 of the human OPG gene, resulting in the production of a huOPG [29-401]-Fc fusion protein:

1436-17:

5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3'
(SEQ ID NO:142)

1436-18:

5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3'
(SEQ ID NO:143)

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The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

5

1436-27:

5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3'

(SEQ ID NO:144)

10 1436-28:

5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3'

(SEQ ID NO:145)

The following primer pairs were used to change the codon for tyrosine residue 28 to phenylalanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc Y28F fusion protein:

15

1436-29:

20 5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3'

(SEQ ID NO:146)

1436-30:

5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3'

25 (SEQ ID NO:147)

The following primer pairs were used to change the codon for proline residue 26 to alanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc P26A fusion protein:

30

1429-83:

5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3

(SEQ ID NO:148)

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1429-84:

5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3'
(SEQ ID NO:149)

5

Each resulting muOPG [22-401]-Fc plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in Example 11.

10

EXAMPLE 8

15

Expression of OPG in E. coli

A. Bacterial Expression VectorspAMG21

20

The expression plasmid pAMG21 can be derived from the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by: (a) destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation; (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites containing the synthetic P_L promoter with a similar fragment obtained from pCFM636 (patent No. 4,710,473) containing the P_L promoter

25

30

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AatII

5' CTAATTCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAAATAAATTCATAT-
 3' TGCAGATTAAGGCGAGAGTGGATGGTTTGTACGGGGGACGTTTTTTATTTAAGTATA-

5 -AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-
 -TTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACGTATTT-

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)

-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

10

ClaI

and then (c) substituting the small DNA sequence between
 the unique *ClaI* and *KpnI* restriction sites with the
 following oligonucleotide:

15

5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC3'
 (SEQ ID NO:48)

20 3' TAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'
 (SEQ ID NO:49)

ClaI

KpnI

The expression plasmid pAMG21 can then be derived from
 pCFM1656 by making a series of site directed base
 25 changes by PCR overlapping oligo mutagenesis and DNA
 sequence substitutions. Starting with the *BglII* site
 (plasmid bp # 180) immediately 5' to the plasmid
 replication promoter *PcopB* and proceeding toward the
 plasmid replication genes, the base pair changes are as
 30 follows:

	<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
	# 204	T/A	C/G
35	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
40	# 994	G/C	A/T
	# 1004	A/T	C/G

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	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
5	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
10	# 2499-2502	AGTG TCAC	GTCA CAGT
	# 2642	TCCGAGC AGGCTCG	7 bp deletion
15	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

20

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

25

(AatII sticky end) (position #4358 in pAMG21) 5' GCGTAACGTATGCATGGTCTCC-
3' TGCACGCATTGCATACGTACCAGAGG-

30 -CCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT-
-GGTACGCTCTCATCCCTTGACGGTCCGTAGTTTATTTTGCTTCCGAGTCAGCTTTCTGA-
-GGGCCTTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTGAGTAGGACAAATCCGC-
35 -CCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCGAGAGGACTCATCCTGTTTAGGCG-
-CGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGC-
-GCCCTCGCCTAAACTTGCAACGCTTCGTTGCCGGGCCTCCACCGCCCGTCTGCGGGCG-
-CATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTCGCT-
40 -GTATTTGACGGTCCGTAGTTTAATTCGTCTTCCGGTAGGACTGCCTACCGGAAAAACGCA-
AatII
-TTCTACAACTCTTTTGTTTATTTTCTAAATACATTCAAATATGGACGTCGTACTTAAC-
45 -AAGATGTTTGAGAAAACAAATAAAAAGATTTATGTAAGTTTATACCTGCAGCATGAATTG-
-TTTTAAAGTATGGGCAATCAATTGCTCCTGTAAATTTGCTTTAGAAATACTTTGGCAGC-
-AAAATTTCATACCCGTTAGTTAACGAGGACAATTTTAACGAAATCTTTATGAAACCGTCG-
-GGTTTGTTGTATTGAGTTTCATTTGCGCATTGGTTAAATGGAAAGTGACCGTGCGCTTAC-
50 -CCAAACAACATAACTCAAAGTAAACGCGTAACCAATTTACCTTTCACTGGCAGCGGAATG-

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-TACAGCCTAATATTTTTGAAATATCCCAAGAGCTTTTTCCTTCGCATGCCACGCTAAAC-
-ATGTCGGATTATAAAAACTTTATAGGGTCTCGAAAAAGGAAGCGTACGGGTGCGATTTG-
5 -ATTCTTTTTCTCTTTTGGTTAAATCGTTGTTTGATTATTTATTTGCTATATTTATTTTTC-
-TAAGAAAAAGAGAAAACCAATTTAGCAACAACTAAATAATAAACGATATAAATAAAAAG-
-GATAATTATCAACTAGAGAAGGAACAATTAATGGTATGTTTCATACACGCATGTAAAAATA-
10 -CTATTAATAGTTGATCTCTTCCTTGTTAATTACCATAACAAGTATGTGCGTACATTTTTAT-
-AACTATCTATATAGTTGCTTTCTCTGAATGTGCAAACTAAGCATTCCGAAGCCATTAT-
-TTGATAGATATATCAACAGAAAGAGACTTACACGTTTTGATTGCTAAGGCTTCGGTAATA-
-TAGCAGTATGAATAGGGAACTAAACCCAGTGATAAGACCTGATGATTTTCGCTTCTTTAA-
15 -ATCGTCATACTTATCCCTTTGATTTGGGTCACTATTCTGGACTACTAAAGCGAAGAAATT-
-TTACATTTGGAGATTTTTTTATTTACAGCATTGTTTTCAAATATATTCCAATTAATCGGTG-
-AATGTAAACCTCTAAAAAATAAATGTCGTAACAAAAGTTTATATAAGGTTAATTAGCCAC-
20 -AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTTTATTAAATTAGCGTCATCAT-
-TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAAATAATTTAATCGCAGTAGTA-
-AATATTGCCTCCATTTTTTAGGGTAATTATCCAGAATTGAAATATCAGATTTAACCATAG-
25 -TTATAACGGAGGTAAAAAATCCCATTAATAGGTCTTAACTTTATAGTCTAAATTGGTATC-
-AATGAGGATAAATGATCGCGAGTAAATAATATTACAATGTACCATTTTAGTCATATCAG-
-TTACTCCTATTTACTAGCGCTCATTTATTATAAGTGTACATGGTAAATCAGTATAGTC-
-ATAAGCATTGATTAATATCATTATTGCTTCTACAGGCTTTAATTTTATTAATTATTCTGT-
30 -TATTCGTAACATAATTATAGTAATAACGAAGATGTCCGAAATTAATAAATTAATAAGACA-
-AAGTGTGCTCGGCATTTATGTCTTTCATACCCATCTCTTTATCCTTACCTATTGTTTGTC-
-TTCACAGCAGCCGTAAATACAGAAAGTATGGGTAGAGAAATAGGAATGGATAACAAACAG-
35 -GCAAGTTTTGCGTGTTATATATCATTAAAACGGTAATAGATTGACATTTGATTCTAATAA-
-CGTTCAAAACGCACAATATATAGTAATTTTGCCATTATCTAACTGTAAACTAAGATTATT-
-ATTGGATTTTTGTCACTATTATATCGCTTGAAATACAATTGTTTAAACATAAGTACCTG-
40 -TAACCTAAAAACAGTGTGATAATATAGCGAAGCTTTATGTTAACAAATTGTATTTCATGGAC-
-TAGGATCGTACAGGTTTACGCAAGAAAATGGTTTGTATAGTCGATTAATCGATTTGATT-
-ATCCTAGCATGTCCAAATGCGTTCTTTTACCAAACAATATCAGCTAATTAGCTAAACTAA-
-CTAGATTTGTTTTAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTGGA-
45 -GATCTAAACAAAATTGATTAATTTCTCTTATTGTATACCAATTGCGCAACCTTAAGCT-
-GCTCACTAGTGTGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAAGAA-
50 -CGAGTGATCACAGCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTTCTT-
-GAAGAAGAAGAAGAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA-
-CTTCTTCTTCTTTTCGGGCTTTCTTTCGACTCAACCGACGACGGTGGCGACTCGTTAT-
-ACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTTGCTGAAAGGAGG-
55 -TGATCGTATTGGGGAACCCCGAGATTGCCCCAGAACTCCCCAAAAACGACTTTCTCTCC-

Sac II

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-AACCGCTCTTCACGCTCTTCACGC 3' [SacII sticky end] (SEQ ID NO:50)
-TTGGCGAGAAGTGCGAGAAGTG 5' (position, #5904 in pAMG21) (SEQ ID NO:46)

5

During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

10

DAMG22-His

The expression plasmid pAMG22-His can be derived from the Amgen expression vector pAMG22 by substituting the small DNA sequence between the unique NdeI (#4795) and EcoRI (#4818) restriction sites of pAMG22 with the following oligonucleotide duplex:

20

	<u>NdeI</u>	<u>NheI</u>	<u>EcoRI</u>
5'	TATGAAACATCATCACCATCACCATCATGCTAGCGTTAACGCGTTGG		3'
	(SEQ ID NO:51)		
3'	ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGCAACCTTAA		5'
	(SEQ ID NO:52)		

MetLysHisHisHisHisHisHisHisAlaSerValAsnAlaLeuGlu
(SEQ ID NO:168)

25

DAMG22

The expression plasmid pAMG22 can be derived from the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473 granted December 1, 1987. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by:

- destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation;
- replacing the DNA sequence between the unique AatII and ClaI restriction sites

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containing the synthetic PL promoter with a similar fragment obtained from pCFM636 (patent No. 4,710,473) containing the PL promoter

5 AatII

5' CTAATTCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAAATAAATTCATAT-
3' TGCAGATTAAGGCGAGAGTGGATGGTTTGTACGGGGGACGTTTTTTATTTAAGTATA-

-AAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-
10 -TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACGTATTT-

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)

-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

Clal

15

and then (c) substituting the small DNA sequence between the unique *Clal* and *KpnI* restriction sites with the following oligonucleotide:

20 5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC 3'
(SEQ ID NO:55)

3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'
(SEQ ID NO:56)

Clal

KpnI

25

The expression plasmid pAMG22 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the *BglII* site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *PcopB* and proceeding toward the plasmid replication genes, the base pair changes are as follows:

30

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	<u>pAMG22 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG22</u>
5	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
10	# 679	G/C	T/A
	# 980	T/A	C/G
	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
15	# 1028	A/T	T/A
	# 1047	C/G	T/A
	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
20	# 2187	C/G	T/A
	# 2480	A/T	T/A
	# 2499-2502	AGTG TCAC	GTCA CAGT
25	# 2642	TCCGAGC AGGCTCG	7 bp deletion
	# 3435	G/C	A/T
30	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end] (position #4358 in pAMG22)

40 5' GCGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAA-
 3' TGCACGCATTGCATACGTACCAGAGGGGTACGCTCTCATCCCTTGACGGTCCGTAGTT-
 -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTGTTTGTCTGGTG-
 45 -TATTTTGCTTCCGAGTCAGCTTTCTGACCCGAAAGCAAAATAGACAACAAACAGCCAC-

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-AACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGG-
 -TTGCGAGAGGACTCATCCTGTTTAGGCGGCCCTCGCCTAAACTTGCAACGCTTCGTTGCC-
 5 -CCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAG-
 -GGGCCTCCACCGCCCGTCTGCGGGCGGTATTTGACGGTCCGTAGTTTAATTCGTCTTC-
 -GCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTGTATTATTTTCTAAAT-
 -CGTAGGACTGCCTACCGGAAAAACGCAAAGATGTTTGAGAAAACAAATAAAAAGATTTA-
 10 AatII
 -ACATTCAAATATGGACGTCTCATAATTTTTAAAAAATTCATTGACAAATGCTAAAATTC-
 -TGTAAGTTTATACCTGCAGAGTATTAAAAATTTTTAAGTAAACTGTTTACGATTTTAAG-
 -TTGATTAATATTCTCAATTGTGAGCGCTCACAATTTATCGATTTGATTCTAGATTTGTTT-
 15 -AACTAATTATAAGAGTTAACTCGCGAGTGTTAAATAGCTAAACTAAGATCTAAACTCA-
 -TAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGAGCTCACTAGTGT-
 -ATTGATTAATTTCTCCTTATTGTATACCAATTGCGCAACCTTAAGCTCGAGTGATCACA-
 20 SacII
 -CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAAGAAGAAGAAGAA-
 -GCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTTCTTCTTCTTCTT-
 -GAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-
 25 -CTTTCGGGCTTTCCTTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATTGG-
 -CCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACCGCTCTTCA-
 -GGAACCCCGGAGATTTGCCCAGAACTCCCCAAAAAACGACTTTCCTCCTTGGCGAGAAGT-
 30 -CGCTCTTCACGC 3' (SEQ ID NO:58)
 -GCGAGAAGTG 5' (SEQ ID NO:57)

[SacII sticky end] (position #5024 in pAMG22)

35 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

40 B. Human OPG Met [32-401]

In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter.
 45 (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120

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(ATCC accession no. 55764). This host has the *lacIQ* promoter and *lacI* gene integrated into a second site in the host chromosome of a prototrophic *E. coli* K12 host. Other commonly used *E. coli* expression vectors and host cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the *luxPR* promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRcCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were phosphorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see Figure 14A) and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-20 and #1257-19 (see above) was directionally inserted between two sites of the plasmid vector pAMG21, namely the NdeI site and BamHI site, using standard recombinant DNA methodology (see Figure 14A and sequences below). The synthetic linker utilized *E. coli* codons and provided for a N-terminal methionine.

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Two clones were selected and plasmid DNA isolated, and the human OPG insert was subsequently DNA sequence confirmed. The resulting pAMG21 plasmid containing amino acids 32-401 of the human OPG polypeptide immediately preceded in frame by a methionine is referred to as pAMG21-huOPG met[32-401] or pAMG21-huOPG met[32-401].

Oligo#1257-19

10 5'-TACGCACTGGATCCTTATAAGCAGCTTATTTTACTGATTGGAC-3'
(SEQ ID NO:59)

Oligo#1257-20

5'-GTCCTCCTGGTACCTACCTAAAACAAC-3' (SEQ ID NO:60)

15

Oligo#1257-21

5'-TATGGATGAAGAACTTCTCATCAGCTGCTGTGTGATAAATGTCC
GCCGGGTAC -3' (SEQ ID NO:61)

20 Oligo#1257-22

5'-CCGGCGGACATTTATCACACAGCAGCTGATGAGAAGTTTCTTCATCCA-3'
(SEQ ID NO:47)

Cultures of pAMG21-huOPG met[32-401] in E. coli GM120 in 2XYT media containing 20 µg/ml kanamycin were incubated at 30°C prior to induction. Induction of huOPG met[32-401] gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and cultures were incubated at either 30°C or 37°C for a further 6 hours. After 6 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then pelleted by

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centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in *E. coli*. Some bacterial pellets were

5 resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and β -mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie stained band of approximately 42kDa was

10 observed on a SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B). The expected gene product would be 370 amino acids in length and have an expected molecular weight of

15 about 42.2 kDa. Following induction at 37°C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or processed by microfluidizing. The pellet processed for microfluidizing was resuspended in 25mM Tris-

20 HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected. An aliquot was removed of the collected sample (microfluidized total lysate), and the remainder was pelleted at 20,000 x g for 20 minutes. The

25 supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea solution (microfluidized insoluble fraction). To an aliquot of either the total soluble, or insoluble

30 fraction was added to an equal volume of 2X Laemalli sample buffer and β -mercaptoethanol to 5% final concentration. The samples were then analyzed by SDS-PAGE. A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the

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insoluble fraction. To purify the recombinant protein inclusion bodies were purified as follows: Bacterial cells were separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This suspension was transferred to a stainless steel cup cooled in ice and subjected to sonic disruption using a Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C. The supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm). Tubes were sealed according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes. The tubes were examined for the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion bodies were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemlli sample

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buffer with 5% β -mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant huOPG[32-401] gene product. The major ~42 kDa band observed after resolving inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira et al. J. Biol. Chem. 262, 10-35 (1987)). The following sequence was determined after 19 cycles:

NH₂ -MDEETSHQLLCDKCPPGTY-COOH (SEQ ID NO:62)

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21 Hu-OPG met[32-401] expression vector, produced by a methionine residue provided by the bacterial expression vector.

C. Human OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see Section B) was cleaved with KpnI and BamHI restriction endonucleases and the resulting fragments were resolved on an agarose gel. The B fragment (~1064 bp fragment) was isolated from the gel using standard methodology. Synthetic oligonucleotides (oligos) #1267-06 and #1267-07 were phosphorylated individually and allowed to form an oligo linker duplex, which contained NdeI and KpnI cohesive ends, using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated oligo linker containing NdeI and KpnI

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cohesive ends and the isolated ~1064 bp fragment of pAMG21-huOP met[32-401] digested with KpnI and BamHI restriction endonucleases were directionally inserted between the NdeI and BamHI sites of pAMG21 using
5 standard recombinant DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of
10 the huOPG-met[22-401] gene.

Oligo #1267-06

5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA
AGA AAC TTC TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG
15 TAC-3' (SEQ ID NO:63)

Oligo #1267-07

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT
CTT CAT CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'
20 (SEQ ID NO:64)

Cultures of pAMG21-huOPG-met[22-401] in E. coli host 393 were placed in 2XYT media containing 20 µg/ml kanamycin and were incubated at 30°C prior to
25 induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml
30 and incubation at either 30°C or 37°C for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation (=30°C I+6 or 37°C I+6). Bacterial cultures were also either pelleted just prior to induction (=30°C PreI) or alternatively no autoinducer

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was added to a separate culture which was allowed to incubate at 30°C for a further 6 hours to give an uninduced (UI) culture (=30°C UI). Bacterial pellets of either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6

5 cultures were resuspended, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Section B. Polyacrylamide gels were either stained with coomassie blue and/or Western transferred to nitrocellulose and immunoprobed with rabbit anti-mu

10 OPG-Fc polyclonal antibody as described in Example 10. The level of gene product following induction compared to either an uninduced (30°C UI) or pre-induction (30°C PreI) sample.

15 D. Murine OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of the murine (mu) OPG (OPG) polypeptide was placed under control of the luxPR promoter in a prokaryotic plasmid expression

20 vector pAMG21 as follows. PCR was performed using oligonucleotides #1257-16 and #1257-15 as primers, plasmid pRcCMV-Mu OPG DNA as a template and thermocycling conditions as described in Section B. The PCR product was purified and cleaved with KpnI and BamHI

25 restriction endonucleases as described in Section B. Synthetic oligos #1260-61 and #1260-82 were phosphorylated individually and allowed to form an oligo linker duplex with NdeI and KpnI cohesive ends using methods described in Section B. The synthetic linker

30 duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1260-61 and #1260-82 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product generated using oligo

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primers #1257-16 and #1257-15 were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation
5 utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG met[22-401] gene.

Expression of recombinant muOPG met[22-401]
10 polypeptide from cultures of 393 cells harboring plasmid pAMG21-MuOPG met[22-401] following induction was determined using methods described in Section C.

Oligo #1257-15
15 5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTC ACG
GAT TGA AC-3' (SEQ ID NO:65)

Oligo #1257-16
5'-GTG CTC CTG GTA CCT ACC TAA AAC AGC ACT GCA CAG
20 TG-3' (SEQ ID NO:66)

Oligo #1260-61
5'-TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA
TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC
25 GGG TAC-3' (SEQ ID NO:67)

Oligo #1260-82
5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CCA-
30 3' (SEQ ID NO:68)

E. Murine OPG met[32-401]

A DNA sequence coding for an N-terminal methionine and amino acids 32 through 401 of murine OPG

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was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. To accomplish this, Synthetic oligos #1267-08 and #1267-09 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[32-401] gene.

Expression of recombinant muOPG-met [32-401] polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.

Oligo #1267-08

5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA
TAA ATG TGC TCC GGG TAC-3' (SEQ ID NO:69)

Oligo #1267-09

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CTG GGT CCA-3' (SEQ ID NO:70)

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E. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal methionine followed by a lysine residue and amino acids 22 through 401 of murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. Synthetic oligos #1282-95 and #1282-96 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1282-95 and #1282-96 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product described in Section D was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG--Met-Lys[22-401] gene.

Expression of recombinant MuOPG Met-Lys[22-401] polypeptide from transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1282-95

5'-TAT GAA AGA AAC TCT GCC TCC AAA ATA CCT GCA TTA
CGA TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC
TCC GGG TAC-3' (SEQ ID NO:71)

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Oligo #1282-96

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT
TCA-3' (SEQ ID NO:72)

5

G. Murine OPG met-lys-(his)₇[22-401]

A DNA sequence coding for N-terminal residues Met-Lys-His-His-His-His-His-His (=MKH) followed by amino acids 22 through 401 of Murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-50 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases and purified. The NdeI and BamHI digested and purified PCR product generated using oligo primers #1300-50 and #1257-15 was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing performed to verify the DNA sequence of the muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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Oligo #1300-50

5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ATC ACC ATC
ATG AAA CTC TGC CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ
ID NO:73)

5

Oligo #1257-15

(see Section D)

H. Murine OPG met-lys[22-401](his)₇

10 A DNA sequence coding for a N-terminal met-
lys, amino acids 22 through 401 murine OPG, and seven
histidine residues following amino acid 401 (=muOPG
MK[22-401]-H7), was placed under control of the lux PR
promoter in prokaryotic expression vector pAMG21 as
15 follows. PCR was performed using oligonucleotides
#1300-49 and #1300-51 as primers and pAMG21-muOPG
met[22-401] DNA as template. Thermocycling conditions
were as described in Section B. The resulting PCR
sample was resolved on an agarose gel, the PCR product
20 was excised, purified, restricted with NdeI and BamHI
restriction endonucleases, and purified. The NdeI and
BamHI digested and purified PCR product was
directionally inserted between the NdeI and BamHI sites
in pAMG21 using standard methodology. The ligation was
25 transformed into E. coli host 393 by electroporation
utilizing the manufacturer's protocol. Clones were
selected, plasmid DNA was isolated, and DNA sequencing
was performed to verify the DNA sequence of the muOPG
MK[22-401]-H7 gene.

30 Expression of the recombinant muOPG
MK-[22-401]-H7 polypeptide from a transformed 393 cells
harboring the recombinant pAMG21 plasmid following
induction was determined using methods described in
Section C.

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Oligo #1300-49

5'-GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT
ACC TGC A-3' (SEQ ID NO:74)

5

Oligo #1300-51

5'-TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGA
TGT AAG CAG CTT ATT TTC ACG GAT TGA ACC TGA TTC CCT A-3'
(SEQ ID NO:75)

10

I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15' as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[27-401] gene.

30

Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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Oligo#1309-74

5'-GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG
AAA CTG GTC AT-3' (SEQ ID NO:76)

5

Oligo#1257-15

(See Section D)

J. Human OPG met[27-401]

10

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-75 and #1309-76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction endonucleases, and purified. The AseI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[27-401] gene.

20

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Expression of the recombinant huOPG-met[27-401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

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Oligo #1309-75

5'-GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA
GAA ACT T-3' (SEQ ID NO:77)

5

Oligo #1309-76

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT
GAT T-3' (SEQ ID NO:78)

10 K. Murine OPG met[22-180]

A DNA sequence coding for a N-terminal methionine and amino acids 22 through 180 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR
15 was performed with oligonucleotides #1309-72 and #1309-73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on
20 an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was
25 directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation was transformed into E. coli host 393 by electroporation
utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[22-180] gene.

30 Expression of recombinant muOPG-met[22-180] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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Oligo #1309-72

5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC
TGC A-3' (SEQ ID NO:79)

5 Oligo #1309-73

5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA
ATT AGC A-3' (SEQ ID NO:80)

L. Murine OPG met[27-180]

10 A DNA sequence coding for a N-terminal
methionine and amino acids 27 through 180 of murine OPG
was placed under the control of the lux PR promoter of
prokaryotic expression vector pAMG21 as follows. PCR
was performed using oligonucleotides #1309-74 (see
15 Section I) and #1309-73 (see Section K) as primers and
plasmid pAMG21-muOPG met[22-401] DNA as template.
Thermocycling conditions were as described in Section B.
The resulting PCR sample was resolved on an agarose gel,
the PCR product excised, purified, restricted with NdeI
20 and BamHI restriction endonucleases, and purified. The
NdeI and BamHI digested and purified PCR product above
was directionally inserted between the NdeI and BamHI
sites in pAMG21 using standard methodology. The
ligation mixture was transformed into E. coli host 393
25 by electroporation utilizing the manufacturer's
protocol. Clones were selected, plasmid DNA was
isolated, and DNA sequencing was performed to verify the
DNA sequence of the muOPG met[27-180] gene.

Expression of recombinant muOPG met[27-180]
30 polypeptide from cultures of transformed 393 cells
harboring the recombinant pAMG21 plasmid following
induction was determined using methods described in
Section C.

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M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker) were phosphorylated individually and allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~413 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of pAMG21-muOPG met[22-401] using standard methodology. Each ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG-met[22-189] or muOPG-met[22-194] genes.

Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

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Oligo #1337-92

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)

Oligo #1337-93

5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)

Oligo #1333-57

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'
(SEQ ID NO:83)

Oligo #1333-58

5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'
(SEQ ID NO:84)15 N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 189, or 27 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers either "muOPG-189 linker" or "muOPG-194 linker" (see Section M) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189] and muOPG met[27-194] following induction of 393 cells

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harboring recombinant pAMG21 plasmids was determined using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

5 A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 185, 22 through 189, or 22 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1331-87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-90 (=huOPG-189 linker), or #1331-91 & #1331-92 (=huOPG-194 linker) were phosphorylated individually and each allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-huOPG-met[27-401] was restricted with KpnI and NdeI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~407 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1331-87 and #1331-88 (huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-189 linker), or oligos #1331-91 and #1331-92 (huOPG-194 linker) [each linker contains NdeI and BamHI cohesive ends], and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[22-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of

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either the huOPG-met[22-185], huOPG-met[22-189], or huOPG-met[22-194] genes.

Expression of recombinant huOPG-met[22-185], huOPG-met[22-189] or huOPG-met[22-194] in transformed
5 393 cells harboring recombinant pAMG21 plasmids following induction was determined using methods described in Section C.

Oligo #1331-87

10 5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)

Oligo #1331-88

5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)

15 Oligo #1331-89

5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)

Oligo #1331-90

20 5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)

Oligo #1331-91

5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT
AAG-3' (SEQ ID NO:89)

25 Oligo #1331-92

5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA
CA-3' (SEQ ID NO:90)

30 P. Human OPG met[27-185], met[27-189], met [27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 185, 27 through 189, or 27 through 194 of the human OPG polypeptide was placed under control of the lux PR

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promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers "huOPG-185 linker", "huOPG-189 linker", or "huOPG-194 linker" (See Section O) each containing NdeI and BamHI cohesive ends, and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases (See Section O) were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[27-401] (See Section J) using standard methodology. Each ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated, and DNA sequencing performed to verify the DNA sequence of either the huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-194] genes.

Expression of recombinant huOPG-met[27-185], huOPG-met[27-189], and huOPG-met[27-194] from recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

O. Murine OPG met[27-401] (P33E, G36S, A45P)

A DNA sequence coding for an N-terminal methionine and amino acids 27 through 48 of human OPG followed by amino acid residues 49 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See Section J) was cleaved with AatII and KpnI restriction endonucleases and a ~1075 bp B fragment isolated from an agarose gel using standard recombinant DNA methodology. Additionally, plasmid pAMG21-muOPG-met[22-401] DNA (See Section D) was digested with KpnI and BamHI restriction

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endonucleases and the ~1064 bp B fragment isolated as described above. The isolated ~1075 bp pAMG21-huOPG-met[27-401] restriction fragment containing AatII & KpnI cohesive ends (see above), the ~1064 bp pAMG21-muOPG-met[22-401] restriction fragment containing KpnI and BamHI sticky ends and a ~5043 bp restriction fragment containing AatII and BamHI cohesive ends and corresponding to the nucleic acid sequence of pAMG21 between AatII & BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, and the presence of the recombinant insert in the plasmid verified using standard DNA methodology. muOPG-27-401 (P33E, G36S, A45P) gene. Amino acid changes in muOPG from proline-33 to glutamic acid-33, glycine-36 to serine-36, and alanine-45 to proline-45, result from replacement of muOPG residues 27 through 48 with huOPG residues 27 through 48.

Expression of recombinant muOPG-met[27-401] (P33E, G36S, A45P) from transformed 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

R. Murine OPG met-lys-(his)₇-ala-ser-(asp)₄-lys[22-401] (A45T)

A DNA sequence coding for an N-terminal His tag and enterokinase recognition sequence which is (NH₂ to COOH terminus): Met-Lys-His-His-His-His-His-His-Ala-Ser-Asp-Asp-Asp-Asp-Lys (=HEK), followed by amino acids 22 through 401 of the murine OPG polypeptide was placed under control of the *lac* repressor regulated Ps4 promoter as follows. pAMG22-His (See Section A) was

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digested with NheI and BamHI restriction endonucleases, and the large fragment (the A fragment) isolated from an agarose gel using standard recombinant DNA methodology. Oligonucleotides #1282-91 and #1282-92 were

5 phosphorylated individually and allowed to form an oligo linker duplex using methods previously described (See Section B). The phosphorylated linker duplex formed between oligos #1282-91 and #1282-92 containing NheI and KpnI cohesive ends, the KpnI and BamHI digested and

10 purified PCR product described (see Section D), and the A fragment of vector pAMG22-His digested with NheI and BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into E. coli host GM120 by electroporation utilizing the

15 manufacturer's protocol. Clones were selected, plasmid DNA isolated and DNA sequencing performed to verify the DNA sequence of the muOPG-HEK[22-401] gene. DNA sequencing revealed a spurious mutation in the natural muOPG sequence that resulted in a single amino acid

20 change of Alanine-45 of muOPG polypeptide to a Threonine.

Expression of recombinant muOPG-HEK[22-401] (A45T) from GM120 cells harboring the recombinant pAMG21 plasmid was determined using methods similar to those

25 described in Section C, except instead of addition of the synthetic autoinducer, IPTG was added to 0.4 mM final to achieve induction.

Oligo #1282-91

30 5'-CTA GCG ACG ACG ACG ACA AAG AAA CTC TGC CTC CAA
AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC ATC AGC TGC TGT
GTG ATA AAT GTG CTC CGG GTA C-3' (SEQ ID NO:91)

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Oligo #1282-92

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT
5 TGT CGT CGT CGT CG-3' (SEQ ID NO:92)

S. Human OPG met-arg-gly-ser-(his)₆[22-401]

Eight oligonucleotides (1338-09 to 1338-16 shown below) were designed to produce a 175 base fragment as overlapping, double stranded DNA. The oligos were annealed, ligated, and the 5' and 3' oligos were used as PCR primers to produce large quantities of the 175 base fragment. The final PCR gene products were digested with restriction endonucleases ClaI and KpnI to yield a fragment which replaces the N-terminal 28 codons of human OPG. The ClaI and KpnI digested PCR product was inserted into pAMG21-huOPG [27-401] which had also been cleaved with ClaI and KpnI. Ligated DNA was transformed into competent host cells of *E. coli* strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Expression of huOPG Met-Arg-Gly-Ser-(His)₆ [22-401] resulting in the formation of large inclusion bodies and the protein was localized to the insoluble (pellet) fraction.

1338-09

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

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1338-10

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC
(SEQ ID NO:94)

5 1338-11

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA GA
(SEQ ID NO:95)

1338-12

10 AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A
(SEQ ID NO:96)

1338-13

TGT TTG GGT ACC CGG CGG GCA TTT GT (SEQ ID NO:97)

15

1338-14

CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC
(SEQ ID NO:98)

20 1338-15

GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T
(SEQ ID NO:99)

1338-16

25 CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT
(SEQ ID NO:100)

T. Human OPG met-lys[22-401] and met-(lys)₃[22-401]

To construct the met-lys and met-(lys)₃

30 versions of human OPG[22-401], overlapping
oligonucleotides were designed to add the appropriate
number of lysine residues. The two oligos for each
construct were designed to overlap, allowing two rounds
of PCR to produce the final product. The template for

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the first PCR reaction was a plasmid DNA preparation containing the human OPG 22-401 gene. The first PCR added the lysine residue(s). The second PCR used the product of the first round and added sequence back to the first restriction site, ClaI.

The final PCR gene products were digested with restriction endonucleases ClaI and KpnI, which replace the N-terminal 28 codons of hu OPG, and then ligated into plasmid pAMG21-hu OPG [27-401] which had been also digested with the two restriction endonucleases. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Neither construct had a detectable level of protein expression and inclusion bodies were not visible. The DNA sequences were confirmed by DNA sequencing.

Oligonucleotide primers to prepare Met-Lys huOPG[22-401]:

1338-17

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT
AAA GGA GGA ATA AAA TG (SEQ ID NO:101)

1338-18

CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA
AAT ATC (SEQ ID NO:102)

1338-20

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TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID
NO:103)

Oligonucleotide primers to prepare Met-(Lys)₃-huOPG[22-
5 401]:

1338-17

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT
AAA GGA GGA ATA AAA TG (SEQ ID NO:104)

10 1338-19

CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC
CTC CAA AAT ATC (SEQ ID NO:105)

1338-20

15 TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID
NO:106)

U. Human and Murine OPG [22-401]/Fc Fusions

Four OPG-Fc fusions were constructed where the
20 Fc region of human IgG1 was fused at the N-terminus of
either human or murine Osteoprotegerin amino acids 22 to
401 (referred to as Fc/OPG [22-401]) or at the C-
terminus (referred to as OPG[22-401]/Fc). Fc fusions
were constructed using the fusion vector pFc-A3
25 described in Example 7.

All fusion genes were constructed using
standard PCR technology. Template for PCR reactions
were plasmid preparations containing the target genes.
Overlapping oligos were designed to combine the
30 C-terminal portion of one gene with the N terminal
portion of the other gene. This process allows fusing
the two genes together in the correct reading frame
after the appropriate PCR reactions have been performed.
Initially one "fusion" oligo for each gene was put into

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a PCR reaction with a universal primer for the vector carrying the target gene. The complimentary "fusion" oligo was used with a universal primer to PCR the other gene. At the end of this first PCR reaction, two
5 separate products were obtained, with each individual gene having the fusion site present, creating enough overlap to drive the second round of PCR and create the desired fusion. In the second round of PCR, the first two PCR products were combined along with universal
10 primers and via the overlapping regions, the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 having been also digested
15 with the two restriction endonucleases. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein
20 expression levels were determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody.

25

Fc/huOPG [22-401]

Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large inclusion
30 bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

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1318-48

CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA
TT (SEQ ID NO:107)

5 1318-49

CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT
GT (SEQ ID NO:108)

Fc/muOPG [22-4011]

10 Expression of the fusion peptide was detected
on a Coomassie stained gel and on a Western blot. The
cells have very large inclusion bodies, and the majority
of the product is in the insoluble (pellet) fraction.
The following primers were used to construct this OPG-Fc
-15 fusion:

1318-50

CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC
TGC (SEQ ID NO:109)

20

1318-51

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT
(SEQ ID NO:110)

25 muOPG [22-4011]/Fc

Expression of the fusion peptide was detected
on a Coomassie stained gel and on a Western blot. The
amount of recombinant product was less than the OPG
fusion proteins having the Fc region in the N terminal
30 position. Obvious inclusion bodies were not detected.
Most of the product appeared to be in the insoluble
(pellet) fraction. The following primers were used to
construct this OPG-Fc fusion:

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1318-54

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC
(SEQ ID NO:111)

5 1318-55

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G
(SEQ ID NO:112)

10 huOPG [22-401]/Fc

Expression of the fusion peptide was not detected on a Coomassie stained gel, although a faint Western positive signal was present. Obvious inclusion bodies were not detected. The following primers were used to prepare this OPG-Fc fusion:

1318-52

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC
(SEQ ID NO:113)

20

1318-53

CAG CTG CAG CTA AGC AGC TTA TTT TTA CTG ATT GG
(SEQ ID NO:114)

25 V. Human OPG met[22-401]-Fc fusion (P25A)

This construct combines a proline to alanine amino acid change at position 25 (P25A) with the huOPG met[22-401]-Fc fusion. The plasmid was digested with restriction endonucleases ClaI and KpnI, which removes the N-terminal 28 codons of the gene, and the resulting small (less than 200 base pair) fragment was gel purified. This fragment containing the proline to alanine change was then ligated into plasmid pAMG21-huOPG [22-401]-Fc fusion which had been digested with

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the two restriction endonucleases. The ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. The expression level of the fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The protein was in the insoluble (pellet) fraction. The cells had large inclusion bodies.

15

W. Human OPG met[22-401] (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 25 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-84 and 1289-85 were annealed to form an oligo linker duplex with XbaI and KpnI cohesive ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker also included an SpeI restriction site which was not present in the original sequence. The linker duplex was directionally inserted between the XbaI and KpnI sites in pAMG21-huOPG-22-401 using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to

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verify the DNA sequence of the HuOPG-Met[22-401] (P25A) gene. The following oligonucleotides were used to generate the XbaI - KpnI linker:

5 Oligo #1289-84

5'-CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC
AAA ATA TCT TCA TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT
GTG TGA TAA ATG TCC GCC GGG TAC -3' (SEQ ID NO:115)

10

Oligo #1289-85

5'- CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC TAG
TTT CTT CAT CAT AAT GAA GAT ATT TTG GAG CAA AAG TTT CCA
TAT GTT ATT CCT CCT T-3' (SEQ ID NO:116)

15

X. Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 26 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-86 and 1289-87 were annealed to form an oligo linker duplex with XbaI and SpeI cohesive ends. The synthetic linker duplex utilized optimal *E. coli* codons and encoded an N-terminal methionine. The linker duplex was directionally inserted between the XbaI and SpeI sites in pAMG21-huOPG[22-401] (P25A) using standard methods. The ligation mixture was introduced into *E. coli* host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] (P26A) gene. One of the clones sequenced was

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found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401] (P26D). The following oligonucleotides were used to generate the XbaI - SpeI linker:

Oligo #1289-86

5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TCC
TGC TAA ATA TCT TCA TTA TGA TGA AGA AA - 3'
(SEQ ID NO:117)

Oligo #1289-87

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA
GGA AAA GTT TCC ATA TGT TAT TCC TCC TT - 3'
(SEQ ID NO:118)

Y. Human OPG met[22-194] (P25A)

20 A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones

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and DNA sequencing was performed to verify the DNA sequence of the huOPG-Met[22-194] (P25A) gene.

EXAMPLE 9

5

Association of OPG Monomers

CHO cells engineered to overexpress muOPG [22-401] were used to generate conditioned media for the analysis of secreted recombinant OPG using rabbit polyclonal anti-OPG antibodies. An aliquot of conditioned media was concentrated 20-fold, then analysed by reducing and non-reducing SDS-PAGE (Figure 15). Under reducing conditions, the protein migrated as a Mr 50-55 kd polypeptide, as would be predicted if the mature product was glycosylated at one or more of its consensus N-linked glycosylation sites. Suprisingly, when the same samples were analysed by non-reducing SDS-PAGE, the majority of the protein migrated as an approximately 100 kd polypeptide, twice the size of the reduced protein. In addition, there was a smaller amount of the Mr 50-55 kd polypeptide. This pattern of migration on SDS-PAGE was consistent with the notion that the OPG product was forming dimers through oxidation of a free sulfhydryl group(s).

25

The predicted mature OPG polypeptide contains 23 cysteine residues, 18 of which are predicted to be involved in forming intrachain disulfide bridges which comprise the four cysteine-rich domains (Figure 12A). The five remaining C-terminal cysteine residues are not involved in secondary structure which can be predicted based upon homology with other TNFR family members. Overall there is a net uneven number of cysteine residues, and it is formally possible that at least one

30

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residue is free to form an intermolecular disulfide bond between two OPG monomers.

To help elucidate patterns of OPG kinesis and monomer association, a pulse-chase labelling study was performed. CHO cells expressing muOPG [22-401] were metabolically labelled as described above in serum-free medium containing ^{35}S -methionine and cysteine for 30 min. After this period, the media was removed, and replaced with complete medium containing unlabelled methionine and cysteine at levels approximately 2,000-fold excess to the original concentration of radioactive amino acids. At 30 min, 1 hr, 2 hr, 4 hr, 6 hr and 12 hr post addition, cultures were harvested by the removal of the conditioned media, and lysates of the conditioned media and adherent monolayers were prepared. The culture media and cell lysates were clarified as described above, and then immunoprecipitated using anti-OPG antibodies as described above. After the immunoprecipitates were washed, they were released by boiling in non-reducing SDS-PAGE buffer then split into two equal halves. To one half, the reducing agent β -mercaptoethanol was added to 5% (v/v) final concentration, while the other half was maintained in non-reducing conditions. Both sets of immunoprecipitates were analysed by SDS-PAGE as described above, then processed for autoradiography and exposed to film. The results are shown in Figure 16. The samples analysed by reducing SDS-PAGE are depicted in the bottom two panels. After synthesis, the OPG polypeptide is rapidly processed to a slightly larger polypeptide, which probably represents modification by N-linked glycosylation. After approximately 1-2 hours, the level of OPG in the cell decreases dramatically, and concomitantly appears in the culture supernatant. This

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appears to be the result of the vectoral transport of OPG from the cell into the media over time, consistent with the notion that OPG is a naturally secreted protein. Analysis of the same immunoprecipitates under
5 nonreducing conditions reveals the relationship between the formation of OPG dimers and secretion into the conditioned media (Figure 16, upper panels). In the first 30-60 minutes, OPG monomers are processed in the cell by apparent glycosylation, followed by dimer
10 formation. Over time, the bulk of OPG monomers are driven into dimers, which subsequently disappear from the cell. Beginning about 60 minutes after synthesis, OPG dimers appear in the conditioned media, and accumulate over the duration of the experiment.
15 Following this period, OPG dimers are formed, which are then secreted into the culture media. OPG monomers persist at a low level inside the cell over time, and small amounts also appear in the media. This does not appear to be the result of breakdown of covalent OPG
20 dimers, but rather the production of sub-stoichiometric amounts of monomers in the cell and subsequent secretion.

Recombinantly produced OPG from transfected CHO cells appears to be predominantly a dimer. To
25 determine if dimerization is a natural process in OPG synthesis, we analysed the conditioned media of a cell line found to naturally express OPG. The CTLL-2 cell line, a murine cytotoxic T lymphocytic cell line (ATCC accession no. TIB-214), was found to express OPG mRNA in
30 a screen of tissue and cell line RNA. The OPG transcript was found to be the same as the cloned and sequenced 2.5-3.0 kb RNA identified from kidney and found to encode a secreted molecule. Western blot analysis of conditioned media obtained from CTLL-2 cells

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shows that most, if not all, of the OPG protein secreted is a dimer (Figure 17). This suggests that OPG dimerization and secretion is not an artifact of overexpression in a cell line, but is likely to be the
5 main form of the product as it is produced by expressing cells.

Normal and transgenic mouse tissues and serum were analysed to determine the nature of the OPG molecule expressed in OPG transgenic mice. Since the
10 rat OPG cDNA was expressed under the control of a hepatocyte control element, extracts made from the parenchyma of control and transgenic mice under non-reducing conditions were analysed (Figure 18). In extract from transgenic, but not control mice, OPG
15 dimers are readily detected, along with substoichiometric amounts of monomers. The OPG dimers and monomers appear identical to the recombinant murine protein expressed in the genetically engineered CHO cells. This strongly suggests that OPG dimers are
20 indeed a natural form of the gene product, and are likely to be key active components. Serum samples obtained from control and transgenic mice were similarly analysed by western blot analysis. In control mice, the majority of OPG protein migrates as a dimer, while small
25 amounts of monomer are also detected. In addition, significant amounts of a larger OPG related protein is detected, which migrates with a relative molecular mass consistent with the predicted size of a covalently-linked trimer. Thus, recombinant OPG is expressed
30 predominantly as a dimeric protein in OPG transgenic mice, and the dimer form may be the basis for the osteopetrotic phenotype in OPG mice. OPG recombinant protein may also exist in higher molecular weight "trimeric" forms.

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To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the murine OPG codons for cysteine residues 195 (C195), C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I sites of the pcDNA 3.1 (+) vector (Invitrogen, San Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent *E. coli* XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

1389-19:

5' -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3'
(SEQ ID NO:150)

1406-38:

5' -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3'
(SEQ ID NO:151)

30

The following primer pairs were used to change the codon for cysteine residue 202 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C202S protein:

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1389-21:

5' -CAC CCT GTC GGA AGA GGC CTT CTT C-3'
(SEQ ID NO:152)

5

1389-22:

5' -GAA GAA GGC CTC TTC CGA CAG GGT G-3' (1389-22)
(SEQ ID NO:153)

10

The following primer pairs were used to change the codon for cysteine residue 277 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C277S protein:

15 1389-23:

5' -TGA CCT CTC GGA AAG CAG CGT GCA-3'
(SEQ ID NO:154)

1389-24:

20 5' -TGC ACG CTG CTT TCC GAG AGG TCA-3'
(SEQ ID NO:155)

The following primer pairs were used to change the codon for cysteine residue 319 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C319S protein:

25

1389-17:

30 5' -CCT CGA AAT CGA GCG AGC AGC TCC-3'
(SEQ ID NO:156)

1389-18:

5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3'
(SEQ ID NO:157)

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The following primer pairs were used to change the codon for cysteine residue 400 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C400S protein:

1406-72:

5' -CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3'
(SEQ ID NO:158)

1406-75:

5' -CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3'
(SEQ ID NO:159)

Each resulting muOPG [22-401] plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. Conditioned media from each transfectant was analysed by non-reducing SDS-PAGE and western blotting with anti-OPG antibodies.

Mutation of any of the five C-terminal cysteine residues results in the production of predominantly (>90%) monomeric 55 kd OPG molecules. This strongly suggests that the C-terminal cysteine residues together play a role in OPG homodimerization.

C-terminal OPG deletion mutants were constructed to map the region(s) of the OPG C-terminal domain which are important for OPG homodimerization. These OPG mutants were constructed by PCR amplification using primers which introduce premature stop translation

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signals in the C-terminal region of murine OPG. The 5' oligo was designed to the MuOPG start codon (containing a HindIII restriction site) and the 3' oligonucleotides (containing a stop codon and XhoI site) were designed to truncate the C-terminal region of muOPG ending at either threonine residue 200 (CT 200), proline 212 (CT212), glutamic acid 293 (CT-293), or serine 355 (CT-355).

The following primers were used to construct muOPG [22-200]:

10

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:160)

15

1391-91;

5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC TTT
TGC GTG GC-3' (1391-91) (SEQ ID NO:161)

20

The following primers were used to construct muOPG [22-212]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:162)

25

1391-90:

5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG AAG
GC -3' (SEQ ID NO:163)

30

The following primers were used to construct muOPG [22-293]:

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1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:164)

1391-89:

5

5'- CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG TGG
CC-3' (SEQ ID NO:165)

10 The following primers were used to construct muOPG
[22-355]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:166)

15

1391-88:

5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-3'
(SEQ ID NO:167)

20 Each resulting muOPG-CT plasmid containing the
appropriate truncation was then transfected into human
293 cells, the mutant OPG-Fc fusion protein purified
from conditioned media as described above. The
biological activity of each protein was assessed the in
25 vitro osteoclast forming assay described in example 11.
The conditioned medias were also analysed by non-
reducing SDS-PAGE and western blotting using anti-OPG
antibodies.

30 Truncation of the C-terminal region of OPG
effects the ability of OPG to form homodimers. CT 355
is predominantly monomeric, although some dimer is
formed. CT 293 forms what appears to be equal molar
amounts of monomer and dimer, and also high molecular

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weight aggregates. However, CT 212 and CT 200 are monomeric.

EXAMPLE 10

Purification of OPG

A. Purification of mammalian OPG-Fc Fusion Proteins

5 L of conditioned media from 293 cells expressing an OPG-Fc fusion protein were prepared as follows. A frozen sample of cells was thawed into 10 ml of 293S media (DMEM-high glucose, 1x L-glutamine, 10% heat inactivated fetal bovine serum (FBS) and 100 ug/ml hygromycin) and fed with fresh media after one day. After three days, cells were split into two T175 flasks at 1:10 and 1:20 dilutions. Two additional 1:10 splits were done to scale up to 200 T175 flasks. Cells were at 5 days post-thawing at this point. Cells were grown to near confluency (about three days) at which time serum-containing media was aspirated, cells were washed one time with 25 ml PBS per flask and 25 ml of SF media (DMEM-high glucose, 1x L-glutamine) was added to each flask. Cells were maintained at 5% CO₂ for three days at which point the media was harvested, centrifuged, and filtered through 0.45m cellulose nitrate filters (Corning).

OPG-Fc fusion proteins were purified using a Protein G Sepharose column (Pharmacia) equilibrated in PBS. The column size varied depending on volume of starting media. Conditioned media prepared as described above was loaded onto the column, the column washed with PBS, and pure protein eluted using 100mM glycine pH 2.7. Fractions were collected into tubes containing 1M Tris pH 9.2 in order to neutralize as quickly as possible. Protein containing fractions were pooled, concentrated

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in either an Amicon Centricon 10 or Centriprep 10 and diafiltered into PBS. The pure protein is stored at -80°C.

5 Murine [22-401]-Fc, Murine [22-180]-Fc, Murine [22-194]-Fc, human [22-401]-Fc and human [22-201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

10 B. Preparation of anti-OPG antibodies

Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG[22-401]-Fc fusion protein. Each rabbit was immunized on day 1 with 50 µg of antigen emulsified in an equal volume of Freund's complete adjuvant. Further boosts (Days 14 and 28) were performed by the same procedure with the substitution of Freund's incomplete adjuvant. Antibody titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal. The sera was first passed over an affinity column to which murine OPG-Fc had been immobilized. The anti-OPG antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc column to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through fractions containing anti-OPG specific antibodies were dialyzed into PBS.

30 C. Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were diafiltered into coupling buffer (0.1M sodium carbonate

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PH 8.3, 0.5M NaCl), and mixed with CNBr-activated
sepharose beads (Pharmacia) for two hours at room
temperature. The resin was then washed with coupling
buffer extensively before blocking unoccupied sites with
5 1M ethanolamine (pH 8.0) for two hours at room
temperature. The resin was then washed with low pH (0.1M
sodium acetate pH 4.0, 0.5M NaCl) followed by a high pH
wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes
were repeated three times. The resin was finally
10 equilibrated with PBS before packing into a column. Once
packed, the resin was washed with PBS. A blank elution
was performed with 0.1M glycine-HCl, pH 2.5), followed
by re-equilibration with PBS.

Concentrated conditioned media from CHO cells
15 expressing muOPG[22-410] was applied to the column at a
low flow rate. The column was washed with PBS until UV
absorbance measured at 280nm returned to baseline. The
protein was eluted from the column first with 0.1M
glycine-HCl (pH 2.5), re-equilibrated with PBS, and
20 eluted with a second buffer (0.1M CAPS, pH 10.5), 1M
NaCl). The two elution pools were diafiltered separately
into PBS and sterile filtered before freezing at -20°C.

Conventional Chromatography

25 CHO cell conditioned media was concentrated
23x in an Amicon spiral wound cartridge (S10Y10) and
diafiltered into 20mM tris pH 8.0. The diafiltered media
was then applied to a Q-sepharose HP (Pharmacia) column
which had been equilibrated with 20mM tris pH 8.0. The
30 column was then washed until absorbance at 280nm reached
baseline. Protein was eluted with a 20 column volume
gradient of 0-300mM NaCl in tris pH 8.0. OPG protein was
detected using a western blot of column fractions.

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Fractions containing OPG were pooled and brought to a final concentration of 300mM NaCl, 0.2mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300mM NaCl, 0.2mM DTT after which the pooled fractions were applied. The column was washed with equilibration buffer until baseline absorbance was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were dialyzed into 10mM potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic hydroxyapatite column (Bio-Rad) which had been equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100mM potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 100-400 mM phosphate.

OPG was detected by coomassie blue staining of SDS-polyacrylamide gels and by western blotting. Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C. However if stored at 4°C in PBS, dimers and what appears to be trimers and tetramers will form after one week.

30

D. Purification of human OPG met[22-401] from E. coli

The bacterial cell paste was suspended into 10 mM EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted

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by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000 x g for one hour at 5°C. The centrifugal pellet was washed by low shear homogenization into water at the original homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH 8.5 at ambient temperature for 30 minutes. This solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first adjustment to pH 4.5 with acetic acid and then chromatography over a column of SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same buffer using 20 column volumes at a flow rate of 0.1 column volumes/minute. The peak fractions containing only the desired OPG form were pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus amebocyte lysate assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluorescence, circular dichroism, differential scanning calorimetry, and protease profiling assays may also be used to examine the folded nature of the protein.

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EXAMPLE 11

Biological Activity of Recombinant OPG

Based on histology and histomorphometry, it
5 appeared that hepatic overexpression of OPG in
transgenic mice markedly decreased the numbers of
osteoclasts leading to a marked increase in bone tissue
(see Example 4). To gain further insight into potential
mechanism(s) underlying this in vivo effect, various
10 forms of recombinant OPG have been tested in an in vitro
culture model of osteoclast formation (osteoclast
forming assay). This culture system was originally
devised by Udagawa (Udagawa et al. Endocrinology 125,
1805-1813 (1989), Proc. Natl. Acad. Sci. USA 87, 7260-
15 7264 (1990)) and employs a combination of bone marrow
cells and cells from bone marrow stromal cell lines. A
description of the modification of this culture system
used for these studies has been previously published
(Lacey et al. Endocrinology 136, 2367-2376 (1995)). In
20 this method, bone marrow cells, flushed from the femurs
and tibiae of mice, are cultured overnight in culture
media (alpha MEM with 10% heat inactivated fetal bovine
serum) supplemented with 500 U/ml CSF-1 (colony
stimulating factor 1, also called M-CSF), a
25 hematopoietic growth factor specific for cells of the
monocyte/macrophage family lineage. Following this
incubation, the non-adherent cells are collected,
subjected to gradient purification, and then cocultured
with cells from the bone marrow cell line ST2 (1×10^6
30 non-adherent cells : 1×10^5 ST2 cells/ ml media). The
media is supplemented with dexamethasone (100 nM) and
the biologically-active metabolite of vitamin D3 known
as 1,25 dihydroxyvitamin D3 (1,25 (OH) $_2$ D3, 10 nM). To
enhance osteoclast appearance, prostaglandin E2 (250 nM)

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is added to some cultures. The coculture period usually ranges from 8 - 10 days and the media, with all of the supplements freshly added, is renewed every 3-4 days. At various intervals, the cultures are assessed for the presence of tartrate acid phosphatase (TRAP) using either a histochemical stain (Sigma Kit # 387A, Sigma, St. Louis, MO) or TRAP solution assay. The TRAP histochemical method allows for the identification of osteoclasts phenotypically which are multinucleated (≥ 3 nuclei) cells that are also TRAP+. The solution assay involves lysing the osteoclast-containing cultures in a citrate buffer (100 mM, pH 5.0) containing 0.1% Triton X-100. Tartrate resistant acid phosphatase activity is then measured based on the conversion of p-nitrophenylphosphate (20 mM) to p-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at RT. The reaction is terminated by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the results are plotted.

Previous studies (Udagawa et al. *ibid*) using the osteoclast forming assay have demonstrated that these cells express receptors for ^{125}I -calcitonin (autoradiography) and can make pits on bone surfaces, which when combined with TRAP positivity confirm that the multinucleated cells have an osteoclast phenotype. Additional evidence in support of the osteoclast phenotype of the multinucleated cells that arise *in vitro* in the osteoclast forming assay are that the cells express αv and $\beta 3$ integrins by immunocytochemistry and calcitonin receptor and TRAP mRNA by *in situ* hybridization (ISH).

The huOPG [22-401]-Fc fusion was purified from CHO cell conditioned media and subsequently utilized in

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the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of TRAP measured in lysed cultures in microtitre plate wells were also inhibited in the presence of OPG with an ID₅₀ of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP cytochemistry (compare Figures 19A-19G and 20).

Purified human IgG1 and TNFbp were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to the OPG portion of the fusion protein. Additional forms of the human and murine molecules have been tested and the cumulative data are summarized in Table 1.

Table 1
Effects of various OPG forms on in vitro osteoclast formation

OPG Construct	Relative Bioactivity in vitro
muOPG [22-401]-Fc	+++
muOPG [22-194]-Fc	+++
muOPG [22-185]-Fc	++
muOPG [22-180]-Fc	-
muOPG [22-401]	+++
muOPG [22-401] C195	+++
muOPG [22-401] C202	+
muOPG [22-401] C277	-
muOPG [22-401] C319	+

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	muOPG [22-401] C400	+
	muOPG [22-185]	-
	muOPG [22-194]	++
	muOPG [22-200]	++
5	muOPG [22-212]	-
	muOPG [22-293]	+++
	muOPG [22-355]	+++
	huOPG [22-401]-Fc	+++
10	huOPG [22-201]-Fc	+++
	huOPG [22-401]-Fc P26A	+++
	huOPG [22-401]-Fc Y28F	+++
	huOPG [22-401]	+++
	huOPG [27-401]-Fc	++
15	huOPG [29-401]-Fc	++
	huOPG [32-401]-Fc	+/-
	+++ , ED ₅₀ = 0.4-2 ng/ml	
	++ , ED ₅₀ = 2-10 ng/ml	
20	+, ED ₅₀ = 10-100 ng/ml	
	-, ED ₅₀ > 100 ng/ml	

25 The cumulative data suggest that murine and human OPG amino acid sequences 22-401 are fully active in vitro, when either fused to the Fc domain, or unfused. They inhibit in a dose-dependent manner and possess half-maximal activities in the 2-10 ng/ml range. Truncation of the murine C-terminus at threonine residue 180 inactivates the molecule, whereas truncations at

30 cysteine 185 and beyond have full activity. The cysteine residue located at position 185 is predicted to form an SS3 bond in the domain 4 region of OPG. Removal of this residue in other TNFR-related proteins has previously been shown to abrogate biological activity

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(Yan et al. J. Biol. Chem. 266, 12099-12104 (1994)).
Our finding that muOPG[22-180]-Fc is inactive while
muOPG[22-185]-Fc is active is consistent with these
findings. This suggests that amino acid residues 22-185
5 define a region for OPG activity.

These findings indicate that like
transgenically-expressed OPG, recombinant OPG protein
also suppressed osteoclast formation as tested in the
osteoclast forming assay. Time course experiments
10 examining the appearance of TRAP+ cells, β 3+ cells,
F480+ cells in cultures continuously exposed to OPG
demonstrate that OPG blocks the appearance TRAP+ and β 3+
cells, but not F480+ cells. In contrast, TRAP+ and β 3+
cells begin to appear as early as day 4 following
15 culture establishment in control cultures. Only F480+
cells can be found in OPG-treated cultures and they
appear to be present at qualitatively the same numbers
as the control cultures. Thus, the mechanism of OPG
effects in vitro appears to involve a blockade in
20 osteoclast differentiation at a step beyond the
appearance of monocyte-macrophages but before the
appearance of cells expressing either TRAP or β 3
integrins. Collectively these findings indicate that
OPG does not interfere with the general growth and
25 differentiation of monocyte-macrophage precursors from
bone marrow, but rather suggests that OPG specifically
blocks the selective differentiation of osteoclasts from
monocyte-macrophage precursors.

To determine more specifically when in the
30 osteoclast differentiation pathway that OPG was
inhibitory, a variation of the in vitro culture method
was employed. This variation, described in (Lacey et
al. supra), employs bone marrow macrophages as
osteoclast precursors. The osteoclast precursors are

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derived by taking the nonadherent bone marrow cells after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000 - 2,000 U/ml CSF-1. Following 4 days of culture, termed the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1. This 2 day period is called the intermediate differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells (1×10^5 cell/ml), dexamethasone (100 nM) and 1,25 (OH) $_2$ D3 (10 nM) are added for the last 8 days for what is termed the terminal differentiation period. Test agents can be added during this terminal period as well. Acquisition of phenotypic markers of osteoclast differentiation are acquired during this terminal period (Lacey et al. ibid).

huOPG [22-401]-Fc (100 ng/ml) was tested for its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP cytochemistry and solution assays were performed. The results of the solution assay are shown in Figure 21. HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases. When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to

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exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL1- α and IL1- β challenge experiments

IL1 increases bone resorption both
5 systemically and locally when injected subcutaneously over the calvaria of mice (Boyce et al., Endocrinology 125, 1142-1150 (1989)). The systemic effects can be assessed by the degree of hypercalcemia and the local effects histologically by assessing the relative
10 magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL1 when injected subcutaneously over the same region of the calvaria as IL1.

IL-1 β experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group: IL1 treated animals (mice
20 received 1 injection/day of 2.5 ug of IL1- β); Low dose muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1 μ g of muOPG [22-401]-Fc); Low dose muopg [22-401]-Fc and IL1- β ; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10
25 μ g muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and IL1- β . All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last
30 injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed.

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After sacrifice the calvaria were removed and processed for paraffin sectioning.

IL1- α experiment

5 Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; IL1 alpha treated animals (mice received 1 injection/day of 5 ug of IL1-alpha); Low dose
10 muOPG [22-401]-Fc treated animals (mice received 1 injection/day of 10 μ g of muOPG [22-401]-Fc; Low dose
muopg [22-401]-Fc and IL1-alpha, (dosing as above); High dose muopg [22-401]-Fc treated animals (mice received 3
15 injections/day of 10 μ g muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and IL1- α . All mice received the same
number of injections/day of either active factor or
20 vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels were measured before the first injection, four hours after the second injection and 24 hours after the third
IL1 injection, just before the animals were sacrificed. The animal weights were measured before the first
25 injection, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

Histological methods

30 Calvarial bone samples were fixed in zinc formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5 μ m thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity

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(Sigma Kit# 387A) and counterstained with hematoxylin. Bone resorption was assessed in the IL1- α treated mice by histomorphometric methods using the Osteomeasure (Osteometrics, Atlanta, GA) by tracing histologic features onto a digitizer platen using a microscope-mounted camera lucida attachment. Osteoclast numbers, osteoclast lined surfaces, and eroded surfaces were determined in the marrow spaces of the calvarial bone. The injected and non-injected sides of the calvaria were measured separately.

Results

IL1- α and IL1- β produced hypercalcemia at the doses used, particularly on the second day, presumably by the induction of increased bone resorption systemically. The hypercalcemic response was blocked by muOPG [22-401]-Fc in the IL1-beta treated mice and significantly diminished in mice treated with IL1-alpha, an effect most apparent on day 2 (Figure 22A-22B).

Histologic analysis of the calvariae of mice treated with IL1-alpha and beta shows that IL1 treatments alone produce a marked increase in the indices of bone resorption including: osteoclast number, osteoclast lined surface, and eroded surface (surfaces showing deep scalloping due to osteoclastic action (Figure 23B, Table 2). In response to IL1- α or IL1- β , the increases in bone resorption were similar on the injected and non-injected sides of the calvaria. Muopg [22-401]-Fc injections reduced bone resorption in both IL1-alpha and beta treated mice and in mice receiving vehicle alone but this reduction was seen only on the muopg [22-401]-Fc injected sides of the calvariae.

The most likely explanation for these observations is that muOPG [22-401]-Fc inhibited bone

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5 resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-401]-Fc injection sites. The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL1-induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

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Table 2. Effects of OPG on variables of bone resorption in IL-1 injected mice.

	Osteoclast Surface % Bone Surface (mean \pm S.D)		Eroded Surface % Bone Surface (mean \pm S.D)		Osteoclast Number/mm ² Tissue Area (mean \pm S.D)	
	Non-injected side	Injected side	Non-injected side	Injected side	Non-injected side	Injected side
Experiment 1						
Control	12.36 \pm 3.44	9.54 \pm 2.46	8.07 \pm 3.90	9.75 \pm 3.16	32.51 \pm 11.09	23.50 \pm 10.83
IL-1- β (2.5 μ g/d)	17.18 \pm 1.30	16.40 \pm 2.16	40.66 \pm 4.28	37.53 \pm 10.28	71.80 \pm 18.76	60.89 \pm 5.16
OPG (40 μ g/d)	10.12 \pm 3.71	5.04 \pm 1.66	9.73 \pm 4.33	4.19 \pm 3.61	32.73 \pm 11.09	15.24 \pm 7.54
OPG+IL-1- β	18.61 \pm 2.46	# 13.26 \pm 2.50	44.87 \pm 8.63	# 25.94 \pm 6.82	69.42 \pm 36.29	# 47.13 \pm 24.26
Experiment 2						
Control	11.56 \pm 4.22	11.95 \pm 2.97	12.67 \pm 5.04	10.03 \pm 5.13	51.72 \pm 23.93	56.03 \pm 30.70
IL-1- α (5 μ g/d)	28.81 \pm 4.84	23.46 \pm 5.76	37.51 \pm 5.16	41.10 \pm 12.53	113.60 \pm 18.04	102.70 \pm 32.09
OPG (40 μ g/d)	14.40 \pm 1.00	# 4.26 \pm 2.54	11.55 \pm 4.14	# 4.29 \pm 3.16	72.28 \pm 14.11	# 22.65 \pm 16.68
OPG+IL-1- α	29.58 \pm 8.80	# 17.83 \pm 3.34	33.66 \pm 9.21	# 24.38 \pm 8.88	146.10 \pm 42.37	# 66.56 \pm 15.62

Different to non-injected side $p < 0.05$ (by paired t test)

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C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range 9.2- 15.7g were divided into groups of ten mice per group. These mice were injected subcutaneously with saline or muOPG [22-401]-Fc 2.5mg/kg *bid* for 14 days (5mg/kg/day). The mice were radiographed before treatment, at day 7 and on day 14. The mice were sacrificed 24 hours after the final injection. The right femur was removed, fixed in zinc formalin, decalcified in formic acid and embedded in paraffin. Sections were cut through the mid region of the distal femoral metaphysis and the femoral shaft. Bone density, by histomorphometry, was determined in six adjacent regions extending from the metaphyseal limit of the growth plate, through the primary and secondary spongiosa and into the femoral diaphysis (shaft). Each region was 0.5 X 0.5 mm².

Radiographic changes

After seven days of treatment there was evidence of a zone of increased bone density in the spongiosa associated with the growth plates in the OPG treated mice relative to that seen in the controls. The effects were particularly striking in the distal femoral and the proximal tibial metaphases (Figure 24A-24B). However bands of increased density were also apparent in the vertebral bodies, the iliac crest and the distal tibia. At 14 days, the regions of opacity had extended further into the femoral and tibial shafts though the intensity of the radio-opacity was diminished. Additionally, there were no differences in the length of the femurs at the completion of the experiment or in the change in length over the duration of the experiment implying that OPG does not alter bone growth.

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Histological Changes

The distal femoral metaphysis showed increased bone density in a regions 1.1 to 2.65 mm in distance from the growth plate (Figures 25 and 26A-26B). This is a region where bone is rapidly removed by osteoclast-mediated bone resorption in mice. In these rapidly growing young mice, the increase in bone in this region observed with OPG treatment is consistent with an inhibition of bone resorption.

D. Effects of Osteoprotegerin on Bone Loss Induced by Ovariectomy in the Rat

Twelve week old female Fisher rats were ovariectomized (OVX) or sham operated and dual xray absorptiometry (DEXA) measurements made of the bone density in the distal femoral metaphysis. After 3 days recovery period, the animals received daily injections for 14 days as follows: Ten sham operated animals received vehicle (phosphate buffered saline); Ten OVX animals received vehicle (phosphate buffered saline); Six OVX animals received OPG-Fc 5mg/kg SC; Six OVX animals received pamidronate (PAM) 5mg/kg SC; Six OVX animals received estrogen (ESTR) 40ug/kg SC. After 7 and 14 days treatment the animals had bone density measured by DEXA. Two days after the last injection the animals were killed and the right tibia and femur removed for histological evaluation.

The DEXA measurements of bone density showed a trend to reduction in the bone density following ovariectomy that was blocked by OPG-Fc. Its effects were similar to the known antiresorptive agents estrogen and pamidronate. (Figure 27). The histomorphometric

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analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.

In vivo Summary

The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in mice treated with IL1- α or IL1- β . The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar to those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on in vitro assays of osteoclast formation, a significant portion of this inhibition is due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

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EXAMPLE 12
Pegylation Derivatives of OPG

5 Preparation of N-terminal PEG-OPG conjugates by
reductive alkylation

HuOPG met [22-194] P25A was buffer exchanged into 25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5 mg/ml. This solution was used to conduct OPG reductive
10 alkylation with monofunctional PEG aldehydes at 5-7 °C. PEG monofunctional aldehydes, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 2-4 moles of PEG aldehyde per mole of OPG.
15 After dissolution of polymer into the protein solution, sodium cyanoborohydride was added to give a final concentration of 15 to 20 mM in the reaction mixture from 1-1.6 M freshly prepared stock solution in cold DI water. The progress of the reaction and the extent of
20 OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO₄, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 16-18 hours, after which the reaction mixture was diluted 6-8 times and the pH
25 lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly-
30 PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE. By N-terminal sequencing, it was determined that the monoPEG-OPG conjugate, the major reaction product in most cases, was 98% N-terminally PEG-modified OPG.

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This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG.

Preparation of PEG-OPG conjugates by acylation

10 HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters, linear
15 or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was
20 monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO₄, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The
25 reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were
30 pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.

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Preparation of dimeric PEG-OPG

HuOPG met [22-194] P25A is prepared for
5 thiolation at 1-3 mg/ml in a phosphate buffer at near
neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA) is
added in a 3-7 fold molar excess while maintaining pH at
7.0 and the rxn stirred at 4°C for 2 hrs. The
monothiolated-OPG is separated from unmodified and
10 polythiolated OPG by ion exchange chromatography and the
protected thiol deprotected by treatment with
hydroxylamine. After deprotection, the hydroxylamine is
removed by gel filtration and the resultant
monothiolated-OPG is subjected to a variety of thiol
15 specific crosslinking chemistries. To generate a
disulfide bonded dimer, the thiolated OPG at >1mg/ml is
allowed to undergo air oxidation by dialysis in slightly
basic phosphate buffer. The covalent thioether OPG
dimer was prepared by reacting the bis-maleimide
20 crosslinker, N,N-bis(3-maleimido propionyl)-2-hydroxy
1,3 propane with the thiolated OPG at >1mg/ml at a 0.6x
molar ratio of crosslinker:OPG in phosphate buffer at pH
6.5. Similarly, the PEG dumbbells are produced by
reaction of substoichiometric amounts of bis-maleimide
25 PEG crosslinkers with thiolated OPG at >1mg/ml in
phosphate buffer at pH 6.5. Any of the above dimeric
conjugates may be further purified using either ion
exchange or size exclusion chromatographies.

Dimeric PEG-OPG conjugates (where OPG is HuOPG met
30 [22-194] P25A prepared using the above procedures
include disulfide-bonded OPG dimer, covalent thioether
OPG dimer with an aliphatic amine type crosslinker, 3.4
kD and 8kD PEG dumbbells and monobells.

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PEG-OPG conjugates were tested for activity in vitro using the osteoclast maturation assay described in Example 11A and for activity in vivo by measuring increased bone density after injection into mice as described in Example 11C. The in vivo activity is shown below in Table 3.

Table 3

In vivo biological activity of Pegylated OPG

10	<u>OPG Construct</u>	<u>Increase in Tibial Bone Density</u>
	muOPG met [22-194]	-
	muOPG met [22-194] 5k PEG	+
15	muOPG met [22-194] 20k PEG	+
	huOPG met [22-194] P25A	-
	huOPG met [22-194] P25A 5k PEG	+
	huOPG met [22-194] P25A 20k PEG	+
20	huOPG met [22-194] P25A 31k PEG	+
	huOPG met [22-194] P25A 57k PEG	+
	huOPG met [22-194] P25A 12k PEG	+
	huOPG met [22-194] P25A 20k Branched PEG	+
	huOPG met [22-194] P25A 8k PEG dimer	+
25	huOPG met [22-194] P25A disulfide crosslink	+

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* * *

While the invention has been described in what
is considered to be its preferred embodiments, it is not
5 to be limited to the disclosed embodiments, but on the
contrary, is intended to cover various modifications and
equivalents included within the spirit and scope of the
appended claims, which scope is to be accorded the
broadest interpretation so as to encompass all such
10 modifications and equivalents.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Amgen Inc.

10

(ii) TITLE OF INVENTION: OSTEOPROTEGERIN

(iii) NUMBER OF SEQUENCES: 168

15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Amgen Inc.

(B) STREET: 1840 Dehavilland Drive

(C) CITY: Thousand Oaks

(D) STATE: California

20

(E) COUNTRY: United States

(F) ZIP: 91320

(v) COMPUTER READABLE FORM:

25

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Winter, Robert B.

(C) REFERENCE/DOCKET NUMBER: A-378-CIP2

40

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 146 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 AAAGGAAGGA AAAAAGCGGC CGCTACANNN NNNNNT

36

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCCACG CGTCCG

16

(2) INFORMATION FOR SEQ ID NO:3:

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGCCGAG GC

12

40

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 147 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAACACGA CGGCCAGT

5

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

20

CAGGAAACAG CTATGACC

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATTAACCC TCACTAAAGG

20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

45

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

- 148 -

GCATTATGAC CCAGAAACCG GAC

23

(2) INFORMATION FOR SEQ ID NO:8:

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGTAGCGCC CTCCTCACA TTC

23

(2) INFORMATION FOR SEQ ID NO:9:

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACTAGTCCC ACAATGAACA AGTGGCTGTG

30

(2) INFORMATION FOR SEQ ID NO:10:

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAGAATGC GGCCGCTAAA CTATGAAACA GCCCAGTGAC CATTG

45

(2) INFORMATION FOR SEQ ID NO:11:

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- 149 -

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
GCCTCTAGAA AGAGCTGGGA C 21
- 15 (2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
20 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
CGCCGTGTTC CATTTATGAG C 21
- 30 (2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
35 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
ATCAAAGGCA GGCATACTT CCTG 24
- 45 (2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
50 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- 150 -

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGCACTCC TGTTTCACGG TCTG

24

10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

15

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAAGACACCT TGAAGGGCCT GATG

24

25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAACTTTTAC AGAAGAGCAT CAGC

24

40

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

45

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

- 151 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCGCGGCCG CATGAACAAG TGGCTGTGCT GCG

33

(2) INFORMATION FOR SEQ ID NO:18:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTCTAGAG AAACAGCCCA GTGACCATTC C

31

25 (2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
30 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40 GTGAAGCTGT GCAAGAACCT GATG

24

(2) INFORMATION FOR SEQ ID NO:20:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 152 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGCA GGGCATACTT CCTG

24

(2) INFORMATION FOR SEQ ID NO:21:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGATCCTGA AGCTGCTCAG TTTG

24

25 (2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
30 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

40 AGCGCGGCCG CGGGGACCAC AATGAACAAG TTG

33

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
45 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 153 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

5 AGCTCTAGAA TTGTGAGGAA ACAGCTCAAT GGC

33

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAGCGGCCG CTGAGCCCAA ATCTTGTGAC AAAACTCAC

39

(2) INFORMATION FOR SEQ ID NO:25:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTAGAGTCG ACTTATCATT TACCCGGAGA CAGGGAGAGG CTCTT

45

(2) INFORMATION FOR SEQ ID NO:26:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 154 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

5 (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

20 CCTCTGCGGC CGCTAAGCAG CTTATTTTCA CGGATTGAAC CTG

43

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

35 CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 155 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCCGTAAGAA ACAGCCCAGT GACC

24

5 (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

20 CCTCTGCGGC CGCTGTTGCA TTTCCTTTCT G

31

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

35

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His
1 5 10 15

Gln Leu Leu

40

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:33:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTCTGCGGC CGCACACACG TTGTCATGTG TTGC

34

25 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
30 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

40 TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:35:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

5

CCTCTGCGGC CGCCTTTTGC GTGGCTTCTC TGTT

34

(2) INFORMATION FOR SEQ ID NO:36:

10

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

40 CCTCTGCGGC CGCTAAGCAG CTTATTTTTA CTGAATGG

38

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCTCTGCGGC CGCCAGGGTA ACATCTATTC CAC

33

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCGAAGCTTC CACCATGAAC AAGTGGCTGT GCTGC

35

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

5 CCTCTGTCGA CTATTATAAG CAGCTTATTT TCACGGATTG

40

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:43:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCTCTGTCGA CTTAACACAC GTTGTCATGT GTTGC

35

40 (2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
45 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCTCTGTGCGA CTTACTTTTG CGTGGCTTCT CTGTT

35

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1537 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTGAAGAGCG TGAAGAGCGG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTTAGA 60

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAACT CAGCTTCCTT 120

TCGGGCTTTC TTCTTCTTCT TCTTCTTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC 180

CTGCAGGTG AACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCTCCTT 240

TAATTAGTTA AAACAAATCT AGAATCAAAT CGATTAATCG ACTATAACAA ACCATTTTCT 300

TGCGTAAACC TGTACGATCC TACAGGTACT TATGTTAAAC AATTGTATTT CAAGCGATAT 360

AATAGTGTGA CAAAAATCCA ATTTATTAGA ATCAAATGTC AATCTATTAC CGTTTTAATG 420

ATATATAACA CGCAAACTT GCGACAAACA ATAGGTAAGG ATAAAGAGAT GGGTATGAAA 480

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GACATAAATG CCGACGACAC TTACAGAATA ATTAATAAAA TTAAAGCCTG TAGAAGCAAT 540
AATGATATTA ATCAATGCTT ATCTGATATG ACTAAAATGG TACATTGTGA ATATTATTTA 600
CTCGCGATCA TTTATCCTCA TTCTATGGTT AAATCTGATA TTTCAATTCT GGATAATTAC 660
CCTAAAAAAT GGAGGCAATA TTATGATGAC GCTAATTTAA TAAAATATGA TCCTATAGTA 720
GATTATTCTA ACTCCAATCA TTCACCGATT AATTGGAATA TATTTGAAAA CAATGCTGTA 780
AATAAAAAAT CTCCAAATGT AATTAAAGAA GCGAAATCAT CAGGTCTTAT CACTGGGTTT 840
AGTTTCCCTA TTCATACTGC TAATAATGGC TTCGGAATGC TTAGTTTTGC ACATTCAGAG 900
AAAGACAAC TATAGATAG TTTATTTTAA CATGCGTGTA TGAACATACC ATTAATTGTT 960
CCTTCTCTAG TTGATAATTA TCGAAAAATA AATATAGCAA ATAATAAATC AAACAACGAT 1020
TTAACCAAAA GAGAAAAAGA ATGTTTAGCG TGGGCATGCG AAGGAAAAAG CTCTTGGGAT 1080
ATTTCAAAAA TATTAGGCTG TAGTAAGCGC ACGGTCACCT TCCATTTAAC CAATGCGCAA 1140
ATGAACTCA ATACAACAAA CCGCTGCCAA AGTATTTCTA AAGCAATTTT AACAGGAGCA 1200
ATTGATTGCC CATACTTTAA AAGTTAAGTA CGACGTCCAT ATTTGAATGT ATTTAGAAAA 1260
ATAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG TCAGGATGGC CTTCTGCTTA 1320
ATTTGATGCC TGGCAGTTTA TGGCGGGCGT CCTGCCCGCC ACCCTCCGGG CCGTTGCTTC 1380
GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCTACTCA GGAGAGCGTT CACCGACAAA 1440
CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT TTCGTTTTAT TTGATGCCTG 1500
GCAGTTCCTT ACTCTCGCAT GGGGAGACCA TGCATAC 1537

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

5 (2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

20 CGATTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGAATTC GGTAC

55

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGAATTCCAA CGCGTTAACC ATATGTTATT CCTCCTTCTA GAATCAAAT

49

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1546 base pairs

(B) TYPE: nucleic acid

40 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

45

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

	GCCTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA	60
5	CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT GTTGTTTGTC GGTGAACGCT	120
	CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCGGA	180
10	GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGCCATC	240
	CTGACGGATG GCCTTTTTGC GTTCTACAA ACTCTTTTGT TTATTTTCT AAATACATTC	300
	AAATATGGAC GTCGTACTTA ACTTTTAAAG TATGGGCAAT CAATTGCTCC TGTAAAATT	360
15	GCTTTAGAAA TACTTTGGCA GCGGTTTGT GTATTGAGTT TCATTGCGC ATTGGTTAAA	420
	TGGAAAGTGA CCGTGCGCTT ACTACAGCCT AATATTTTG AAATATCCCA AGAGCTTTTT	480
20	CCTTCGCATG CCCACGCTAA ACATTCTTTT TCTCTTTTGG TTAAATCGTT GTTTGATTTA	540
	TTATTTGCTA TATTTATTTT TCGATAATTA TCAACTAGAG AAGGAACAAT TAATGGTATG	600
	TTCATACACG CATGTAAAAA TAACTATCT ATATAGTTGT CTTTCTCTGA ATGTGCAAAA	660
25	CTAAGCATT CGAAGCCATT ATTAGCAGTA TGAATAGGGA AACTAAACCC AGTGATAAGA	720
	CCTGATGATT TCGCTTCTTT AATTACATTT GGAGATTTTT TATTACAGC ATTGTTTTCA	780
30	AATATATTCC AATTAATCGG TGAATGATTG GAGTTAGAAT AATCTACTAT AGGATCATAT	840
	TTTATTAAAT TAGCGTCATC ATAATATTGC CTCCATTTTT TAGGGTAATT ATCCAGAATT	900
	GAAATATCAG ATTTAACCAT AGAATGAGGA TAAATGATCG CGAGTAAATA ATATTCACAA	960
35	TGTACCATT TAGTCATATC AGATAAGCAT TGATTAATAT CATTATTGCT TCTACAGGCT	1020
	TTAATTTTAT TAATTATTCT GTAAGTGTCG TCGGCATTTA TGTCTTTCAT ACCCATCTCT	1080
40	TTATCCTTAC CTATTGTTTG TCGCAAGTTT TCGGTGTTAT ATATCATTAA AACGGTAATA	1140
	GATTGACATT TGATTCTAAT AAATTGGATT TTTGTCACAC TATTATATCG CTTGAAATAC	1200
	AATTGTTTAA CATAAGTACC TGTAGGATCG TACAGGTTTA CGCAAGAAAA TGGTTTGTTA	1260
45	TAGTCGATTA ATCGATTGA TTCTAGATTT GTTTTAACTA ATTAAAGGAG GAATAACATA	1320
	TGGTTAACGC GTTGGAATTC GAGCTCACTA GTGTCGACCT GCAGGGTACC ATGGAAGCTT	1380
50	ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC CCGAAAGGAA GCTGAGTTGG	1440
	CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCTTGG GGCCTCTAAA CGGGTCTTGA	1500

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GGGGTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT TCACGC

1546

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 47 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATGAAACAT CATCACCATC ACCATCATGC TAGCGTTAAC GCGTTGG

47

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AATTCCAACG CGTTAACGCT AGCATGATGG TGATGGTGAT GATGTTTCA

49

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 141 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

5 CTAATTCCGC TCTCACCTAC CAAACAATGC CCCCCTGCAA AAAATAAATT CATATAAAAA 60
ACATACAGAT AACCATCTGC GGTGATAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC 120
TGGCGGTGAT ACTGAGCACA T 141

10 (2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 147 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

25 CGATGTGCTC AGTATCACCG CCAGTGGTAT TTATGTCAAC ACCGCCAGAG ATAATTTATC 60
ACCGCAGATG GTTATCTGTA TGTTTTTTAT ATGAATTTAT TTTTGCAGG GGGGCATTGT 120
TTGGTAGGTG AGAGCGGAAT TAGACGT 147

30

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

45

CGATTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGAATTC GGTAC 55

50

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(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATTCCAA CGCGTTAACC ATAIGTTATT CTCCTTCTA GAATCAAAT

49

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 668 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GTGAAGAGCG TGAAGAGCGG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTTAGA 60
GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAACT CAGCTTCCTT 120
TCGGGCTTTC TTCTTCTTCT TCTTCTTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC 180
CTGCAGGTCG ACACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCTCCTT 240
TAATTAGTTA ACTCAAATCT AGAATCAAAT CGATAAATTG TGAGCGCTCA CAATTGAGAA 300
TATTAATCAA GAATTTTAGC ATTTGTCAAA TGAATTTTTT AAAAATTATG AGACGTCCAT 360
ATTTGAATGT ATTTAGAAAA ATAAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG 420
TCAGGATGGC CTTCTGCTTA ATTTGATGCC TGGCAGTTTA TGGCGGGCGT CCTGCCCGCC 480
ACCCTCCGGG CCGTTGCTTC GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCTACTCA 540
GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTC TC GACTGAGCCT 600

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TTCGTTTTAT TTGATGCCTG GCAGTTCCT ACTCTCGCAT GGGGAGACCA TGCATACGTT 660
ACGCACGT 668

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 726 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CGGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA 60
CGAAAGGCTC AGTCGAAAGA CTGGGCCCTT CGTTTTATCT GTTGTTTGTC GGTGAACGCT 120
CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTGAACG TTGCGAAGCA ACGGCCCCGA 180
GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGGGCCT 240
CCCACGCCC GTCTGCGGG CGGTATTTGA CGGTCCGTAG TTTAATTCGT CTTGCCATC 300
CTGACGGATG GCCTTTTTGC GTTTCTACAA ACTCTTTTGT TTATTTTCT AAATACATTC 360
AAATATGGAC GTCTCATAAT TTTTAAAAA TTCATTTGAC AAATGCTAAA ATTCTTGATT 420
AATATTCTCA ATTGTGAGCG CTCACAATT ATCGATTGA TTCTAGATT GTTTTAACTA 480
ATTAAAGGAG GAATAACATA TGGTTAACGC GTTGAATTC GAGCTCACTA GTGTCGACCT 540
GCAGGGTACC ATGGAAGCTT ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC 600
CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG 660
GGCCTCTAAA CGGGTCTTGA GGGGTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT 720
TCACGC 726

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(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT GGAC

44

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTCCTCCTGG TACCTACCTA AAACAAC

27

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTACCCGGCG

60

GACATTTATC ACACAGCAGC TGATGAGAAG TTTCTTCATC CA

102

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(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

15 Met Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro
1 5 10 15
Gly Thr Tyr

20

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

35

TATGGAAACT TTTCCTCCAA AATATCTTCA TTATGATGAA GAACTTCTC ATCAGCTGCT 60
GTGTGATAAA TGTCCGCCGG GTAC 84

40 (2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

5 CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCATAA TGAAGATATT 60
TTGGAGGAAA AGTTTCCA 78

(2) INFORMATION FOR SEQ ID NO:65:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTCACGGATT GAAC 44

25 (2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

40 GTGCTCCTGG TACCTACCTA AAACAGCACT GCACAGTG 38

(2) INFORMATION FOR SEQ ID NO:67:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:
TATGGAAACT CTGCCTCCAA AATACCTGCA TTACGATCCG GAAACTGGTC ATCAGCTGCT 60
GTGTGATAAA TGTGCTCCGG GTAC 84
- 10 (2) INFORMATION FOR SEQ ID NO:68:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
15 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
20
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:
25 CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT 60
TTGGAGGCAG AGTTTCCA 78
- 30 (2) INFORMATION FOR SEQ ID NO:69:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
35 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
40
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:
TATGGACCCA GAAACTGGTC ATCAGCTGCT GTGTGATAAA TGTGCTCCGG GTAC 54
- 45 (2) INFORMATION FOR SEQ ID NO:70:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
50 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

10

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC TGGGTCCA

48

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 87 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

25

TATGAAAGAA ACTCTGCCTC CAAAATACCT GCATTACGAT CCGGAAACTG GTCATCAGCT

60

GCTGTGTGAT AAATGTGCTC CGGGTAC

87

30

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

35

(A) LENGTH: 81 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

45

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT

60

TTGGAGGCAG AGTTTCTTTC A

81

50

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(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GTTCTCCTCA TATGAAACAT CATCACCATC ACCATCATGA AACTCTGCCT CAAAATACC 60
TGCATTACGA T 71

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTTCTCCTCA TATGAAAGAA ACTCTGCCTC CAAAATACCT GCA 43

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TACGCACTGG ATCCTTAATG ATGGTGATGG TGATGATGTA AGCAGCTTAT TTTCACGGAT 60
5 TGAACCTGAT TCCCTA 76

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTTCTCCTCA TATGAAATAC CTGCATTACG ATCCGGAAC TGGTCAT 47

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

35 GTTCTCCTAT TAATGAAATA TCTTCATTAT GATGAAGAAA CTT 43

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

5 TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT 40

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

20 GTTCTCCTCA TATGGAAACT CTGCCTCCAA AATACCTGCA 40

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

40 TACGCACTGG ATCCTTATGT TGCATTTCTT TTCTGAATTA GCA 43

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

5 CCGGAAACAG ATAATGAG

18

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GATCCTCATT ATCTGTTT

18

(2) INFORMATION FOR SEQ ID NO:83:

25

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

40 CCGGAAACAG AGAAGCCACG CAAAAGTAAG

30

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

5 GATCCTTACT TTTGCGTGGC TTCTCTGTTT

30

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATGTTAATG AG

12

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GATCCTCATT AACA

14

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

5 TATGTTCCGG AAACAGTTAA G

21

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

25 GATCCTTAAC TGTTTCCGGA ACA

23

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TATGTTCCGG AAACAGTGAA TCAACTCAAA AATAAG

36

(2) INFORMATION FOR SEQ ID NO:90:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:
GATCCTTATT TTTGAGTTGA TTCACTGTTT CCGGAACA 38

10 (2) INFORMATION FOR SEQ ID NO:91:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:
25 CTAGCGACGA CGACGACAAA GAAACTCTGC CTCCAAAATA CCTGCATTAC GATCCGGAAA 60
CTGGTCATCA GCTGCTGTGT GATAAATGTG CTCCGGGTAC 100

30 (2) INFORMATION FOR SEQ ID NO:92:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 base pairs
(B) TYPE: nucleic acid
35 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:
45 CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT 60
TTGGAGGCAG AGTTTCTTTG TCGTCGTCGT CG 92

50

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(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ACAAACACAA TCGATTGAT ACTAGA

26

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTTGTTTAA CTAATTAAAG GAGGAATAAA ATATGAGAGG ATCGCATCAC

50

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

CATCACCATC ACGAAACCTT CCCGCCGAAA TACCTGCACT ACGACGAAGA

50

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(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

AACCTCCAC CAGCTGCTGT GCGACAAATG CCCGCCGGGT ACCCAAACA

49

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTTTGGGTA CCCGGCGGGC ATTTGT

26

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CGCACAGCAG CTGGTGGGAG GTTCTTCGT CGTAGTGCAG GTATTTCGGC

50

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(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGAAGGTTT CGTGATGGTG ATGGTGATGC GATCCTCTCA TATTTTATT

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CCTCCTTTAA TTAGTTAAAA CAAATCTAGT ATCAAATCGA TTGTGTTTGT

(2) INFORMATION FOR SEQ ID NO:101:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

ACAAACACAA TCGATTGAT ACTAGATTG TTTTAACTAA TTAAAGGAGG AATAAAATG

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(2) INFORMATION FOR SEQ ID NO:102:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

CTAATTAAAG GAGGAATAAA ATGAAAGAAA CTTTTCCTCC AAAATATC

48

(2) INFORMATION FOR SEQ ID NO:103:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C

31

(2) INFORMATION FOR SEQ ID NO:104:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

ACAAACACAA TCGATTTGAT ACTAGATTTG TTTTAACTAA TTAAAGGAGG AATAAAATG

59

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(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

CTAATTAAAG GAGGAATAAA ATGAAAAAAA AAGAACTTT TCCTCCAAAA TATC

54

(2) INFORMATION FOR SEQ ID NO:106:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C

31

(2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

CAGCCCGGGT AAAATGGAAA CGTTTCCTCC AAAATATCTT CATT

44

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(2) INFORMATION FOR SEQ ID NO:108:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

CGTTTCCATT TTACCCGGGC TGAGCGAGAG GCTCTTCTGC GTGT

44

(2) INFORMATION FOR SEQ ID NO:109:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

CGCTCAGCCC GGGTAAAATG GAAACGTTGC CTCCAAATA CCTGC

45

(2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

CCATTTTACC CGGGCTGAGC GAGAGGCTCT TCTGCGTGT

39

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(2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

(2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

CAGCTGCAGC TAAGCAGCTT ATTTTCACGG ATTG

34

(2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

AAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

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(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

CAGCTGCAGC TAAGCAGCTT ATTTTACTG ATTGG

35

(2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

CTAGAAGGAG GAATAACATA TGGAACTTT TGCTCCAAA TATCTTCATT ATGATGAAGA

60

AACTAGTCAT CAGCTGCTGT GTGATAAATG TCCGCCGGGT AC

102

(2) INFORMATION FOR SEQ ID NO:116:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 94 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GACTAGTTTC TTCATCATAA TGAAGATATT 60
5 TTGGAGCAAA AGTTTCCATA TGTTATTCCT CCTT 94

(2) INFORMATION FOR SEQ ID NO:117:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

CTAGAAGGAG GAATAACATA TGGAACTTT TCCTGCTAAA TATCTTCATT ATGATGAAGA 60
AA 62

(2) INFORMATION FOR SEQ ID NO:118:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

CTAGTTTCTT CATCATAATG AAGATATTTA GCAGGAAAAG TTTCCATATG TTATTCCTCC 60
TT 62

45 (2) INFORMATION FOR SEQ ID NO:119:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

Tyr His Tyr Tyr Asp Gln Asn Gly Arg Met Cys Glu Glu Cys His Met
 1 5 10 15
 Cys Gln Pro Gly His Phe Leu Val Lys His Cys Lys Gln Pro Lys Arg
 20 25 30
 Asp Thr Val Cys His Lys Pro Cys Glu Pro Gly Val Thr Tyr Thr Asp
 35 40 45
 Asp Trp His
 50

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2432 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 124...1326

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

ATCAAAGGCA GGGCATACTT CCTGTTGCCC AGACCTTATA TAAAACGTCA TGTTGCGCTG 60
 GGCAGCAGAG AAGCACCTAG CACTGGCCCA GCGGCTGCCG CCTGAGGTTT CCAGAGGACC 120
 ACA ATG AAC AAG TGG CTG TGC TGT GCA CTC CTG GTG TTC TTG GAC ATC 168
 Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile
 1 5 10 15
 ATT GAA TGG ACA ACC CAG GAA ACC TTT CCT CCA AAA TAC TTG CAT TAT 216
 Ile Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr
 20 25 30

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	GAC CCA GAA ACC GGA CGT CAG CTC TTG TGT GAC AAA TGT GCT CCT GGC Asp Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly	264
	35 40 45	
5	ACC TAC CTA AAA CAG CAC TGC ACA GTC AGG AGG AAG ACA CTG TGT GTC Thr Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val	312
	50 55 60	
10	CCT TGC CCT GAC TAC TCT TAT ACA GAC AGC TGG CAC ACG AGT GAT GAA Pro Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu	360
	65 70 75	
15	TGC GTG TAC TGC AGC CCC GTG TGC AAG GAA CTG CAG ACC GTG AAA CAG Cys Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln	408
	80 85 90 95	
20	GAG TGC AAC CGC ACC CAC AAC CGA GTG TGC GAA TGT GAG GAA GGG CGC Glu Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg	456
	100 105 110	
25	TAC CTG GAG CTC GAA TTC TGC TTG AAG CAC CGG AGC TGT CCC CCA GGC Tyr Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly	504
	115 120 125	
30	TTG GGT GTG CTG CAG GCT GGG ACC CCA GAG CGA AAC ACG GTT TGC AAA Leu Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys	552
	130 135 140	
35	AGA TGT CCG GAT GGG TTC TTC TCA GGT GAG ACG TCA TCG AAA GCA CCC Arg Cys Pro Asp Gly Phe Ser Ser Gly Glu Thr Ser Ser Lys Ala Pro	600
	145 150 155	
40	TGT AGG AAA CAC ACC AAC TGC AGC TCA CTT GGC CTC CTG CTA ATT CAG Cys Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln	648
	160 165 170 175	
45	AAA GGA AAT GCA ACA CAT GAC AAT GTA TGT TCC GGA AAC AGA GAA GCA Lys Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala	696
	180 185 190	
50	ACT CAA AAT TGT GGA ATA GAT GTC ACC CTG TGC GAA GAG GCA TTC TTC Thr Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe	744
	195 200 205	
55	AGG TTT GCT GTG CCT ACC AAG ATT ATA CCG AAT TGG CTG AGT GTT CTG Arg Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu	792
	210 215 220	
60	GTG GAC AGT TTG CCT GGG ACC AAA GTG AAT GCA GAG AGT GTA GAG AGG Val Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg	840
	225 230 235	

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5	ATA AAA CGG AGA CAC AGC TCG CAA GAG CAA ACT TTC CAG CTA CTT AAG	888
	Ile Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys	
	240 245 250 255	
10	CTG TGG AAG CAT CAA AAC AGA GAC CAG GAA ATG GTG AAG AAG ATC ATC	936
	Leu Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile	
	260 265 270	
15	CAA GAC ATT GAC CTC TGT GAA AGC AGT GTG CAA CGG CAT ATC GGC CAC	984
	Gln Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His	
	275 280 285	
20	GCG AAC CTC ACC ACA GAG CAG CTC CGC ATC TTG ATG GAG AGC TTG CCT	1032
	Ala Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro	
	290 295 300	
25	GGG AAG AAG ATC AGC CCA GAC GAG ATT GAG AGA ACG AGA AAG ACC TGC	1080
	Gly Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys	
	305 310 315	
30	AAA CCC AGC GAG CAG CTC CTG AAG CTA CTG AGC TTG TGG AGG ATC AAA	1128
	Lys Pro Ser Glu Gln Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys	
	320 325 330 335	
35	AAT GGA GAC CAA GAC ACC TTG AAG GGC CTG ATG TAC GCA CTC AAG CAC	1176
	Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His	
	340 345 350	
40	TTG AAA GCA TAC CAC TTT CCC AAA ACC GTC ACC CAC AGT CTG AGG AAG	1224
	Leu Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys	
	355 360 365	
45	ACC ATC AGG TTC TTG CAC AGC TTC ACC ATG TAC CGA TTG TAT CAG AAA	1272
	Thr Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys	
	370 375 380	
50	CTC TTT CTA GAA ATG ATA GGG AAT CAG GTT CAA TCA GTG AAG ATA AGC	1320
	Leu Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser	
	385 390 395	
55	TGC TTA TAGTTAGGAA TGGTCACTGG GCTGTTTCTT CAGGATGGGC CAACACTGAT	1376
	Cys Leu	
	400	
60	GGAGCAGATG GCTGCTTCTC CGGCTCTTGA AATGGCAGTT GATTCCTTTC TCATCAGTTG	1436
	GTGGGAATGA AGATCCTCCA GCCCAACACA CACACTGGGG AGTCTGAGTC AGGAGAGTGA	1496
65	GGCAGGCTAT TTGATAATTG TGCAAAGCTG CCAGGTGTAC ACCTAGAAAG TCAAGCACCC	1556

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TGAGAAAGAG GATATTTTAA TAACCTCAAA CATAGGCCCT TTCCTTCCTC TCCTTATGGA 1616
 TGAGTACTCA GAAGGCTTCT ACTATCTTCT GTGTCATCCC TAGATGAAGG CCTCTTTTAT 1676
 5 TTATTTTTTT ATTCTTTTTT TCGGAGCTGG GGACCGAACC CAGGGCCTTG CGCTTGCGAG 1736
 GCAAGTGCTC TACCACTGAG CTAAATCTCC AACCCTGAA GGCCTCTTTC TTTCTGCCTC 1796
 10 TGATAGTCTA TGACATTCTT TTTTCTACAA TTCGTATCAG GTGCACGAGC CTTATCCCAT 1856
 TTGTAGGTTT CTAGGCAAGT TGACCGTTAG CTATTTTCC CTCTGAAGAT TTGATTGAG 1916
 TTGCAGACTT GGCTAGACAA GCAGGGGTAG GTTATGGTAG TTTATTTAAC AGACTGCCAC 1976
 15 CAGGAGTCCA GTGTTTCTTG TTCCTCTGTA GTGTACCTA AGCTGACTCC AAGTACATTT 2036
 AGTATGAAAA ATAATCAACA AATTTTATTC CTTCTATCAA CATTGGCTAG CTTTGTTTCA 2096
 20 GGGCACTAAA AGAACTACT ATATGGAGAA AGAATTGATA TTGCCCCCAA CGTTCAACAA 2156
 CGCAATAGTT TATCCAGCTG TCATGCCTGG TTCAGTGTCT ACTGACTATG CGCCCTCTTA 2216
 TTAGTGATG CAGTAATTCA ACTGGAAATA GTAATAATAA TAATAGAAAT AAAATCTAGA 2276
 25 CTCCATTGGA TCTCTCTGAA TATGGGAATA TCTAACTTAA GAAGCTTTGA GATTTGAGTT 2336
 GTGTTAAAGG CTTTTATTAA AAAGCTGATG CTCTTCTGTA AAAGTTACTA ATATATCTGT 2396
 30 AAGACTATTA CAGTATTGCT ATTTATATCC ATCCAG 2432

(2) INFORMATION FOR SEQ ID NO:121:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 401 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile
 1 5 10 15
 50 Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
 20 25 30

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Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
 35 40 45

5 Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
 50 55 60

Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
 65 70 75 80

10 Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu
 85 90 95

15 Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
 100 105 110

Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu
 115 120 125

20 Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
 130 135 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
 145 150 155 160

25 Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln Lys
 165 170 175

30 Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
 180 185 190

Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
 195 200 205

35 Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val
 210 215 220

Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
 225 230 235 240

40 Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
 245 250 255

45 Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln
 260 265 270

Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His Ala
 275 280 285

50 Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro Gly
 290 295 300

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Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys
 305 310 315 320
 5 Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
 325 330 335
 Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu
 340 345 350
 10 Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr
 355 360 365
 15 Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu
 370 375 380
 Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
 385 390 395 400
 20 Leu

(2) INFORMATION FOR SEQ ID NO:122:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1324 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 90..1292

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

CCTTATATAA ACGTCATGAT TGCCTGGGCT GCAGAGACGC ACCTAGCACT GACCCAGCGG 60
 CTGCCTCCTG AGGTTTCCCG AGGACCACA ATG AAC AAG TGG CTG TGC TGC GCA 113
 45 Met Asn Lys Trp Leu Cys Cys Ala
 1 5
 CTC CTG GTG CTC CTG GAC ATC ATT GAA TGG ACA ACC CAG GAA ACC CTT 161
 50 Leu Leu Val Leu Leu Asp Ile Ile Glu Trp Thr Thr Gln Glu Thr L u
 10 15 20

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	CCT CCA AAG TAC TTG CAT TAT GAC CCA GAA ACT GGT CAT CAG CTC CTG	209
	Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His Gln Leu Leu	
	25 30 35 40	
5	TGT GAC AAA TGT GCT CCT GGC ACC TAC CTA AAA CAG CAC TGC ACA GTG	257
	Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Val	
	45 50 55	
10	AGG AGG AAG ACA TTG TGT GTC CCT TGC CCT GAC CAC TCT TAT ACG GAC	305
	Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser Tyr Thr Asp	
	60 65 70	
15	AGC TGG CAC ACC AGT GAT GAG TGT GTG TAT TGC AGC CCA GTG TGC AAG	353
	Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro Val Cys Lys	
	75 80 85	
20	GAA CTG CAG TCC GTG AAG CAG GAG TGC AAC CGC ACC CAC AAC CGA GTG	401
	Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val	
	90 95 100	
25	TGT GAG TGT GAG GAA GGG CGT TAC CTG GAG ATC GAA TTC TGC TTG AAG	449
	Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys	
	105 110 115 120	
30	CAC CGG AGC TGT CCC CCG GGC TCC GGC GTG GTG CAA GCT GGA ACC CCA	497
	His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala Gly Thr Pro	
	125 130 135	
35	GAG CGA AAC ACA GTT TGC AAA AAA TGT CCA GAT GGG TTC TTC TCA GGT	545
	Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe Phe Ser Gly	
	140 145 150	
40	GAG ACT TCA TCG AAA GCA CCC TGT ATA AAA CAC ACG AAC TGC AGC ACA	593
	Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn Cys Ser Thr	
	155 160 165	
45	TTT GGC CTC CTG CTA ATT CAG AAA GGA AAT GCA ACA CAT GAC AAC GTG	641
	Phe Gly Leu Leu Leu Ile Gln Lys Gly Asn Ala Thr His Asp Asn Val	
	170 175 180	
50	TGT TCC GGA AAC AGA GAA GCC ACG CAA AAG TGT GGA ATA GAT GTC ACC	689
	Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile Asp Val Thr	
	185 190 195 200	
55	CTG TGT GAA GAG GCC TTC TTC AGG TTT GCT GTT CCT ACC AAG ATT ATA	737
	Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Ile Ile	
	205 210 215	

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	CCA AAT TGG CTG AGT GTT TTG GTG GAC AGT TTG CCT GGG ACC AAA GTG	785
	Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly Thr Lys Val	
	220 225 230	
5	AAT GCC GAG AGT GTA GAG AGG ATA AAA CGG AGA CAC AGC TCA CAA GAG	833
	Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser Ser Gln Glu	
	235 240 245	
10	CAA ACC TTC CAG CTG CTG AAG CTG TGG AAA CAT CAA AAC AGA GAC CAG	881
	Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Arg Asp Gln	
	250 255 260	
15	GAA ATG GTG AAG AAG ATC ATC CAA GAC ATT GAC CTC TGT GAA AGC AGC	929
	Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Ser Ser	
	265 270 275 280	
20	GTG CAG CGG CAT CTC GGC CAC TCG AAC CTC ACC ACA GAG CAG CTT CTT	977
	Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu Gln Leu Leu	
	285 290 295	
	GCC TTG ATG GAG AGC CTG CCT GGG AAG AAG ATC AGC CCA GAA GAG ATT	1025
	Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro Glu Glu Ile	
	300 305 310	
25	GAG AGA ACG AGA AAG ACC TGC AAA TCG AGC GAG CAG CTC CTG AAG CTA	1073
	Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu Leu Lys Leu	
	315 320 325	
30	CTC AGT TTA TGG AGG ATC AAA AAT GGT GAC CAA GAC ACC TTG AAG GGC	1121
	Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly	
	330 335 340	
35	CTG ATG TAT GCC CTC AAG CAC TTG AAA ACA TCC CAC TTT CCC AAA ACT	1169
	Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe Pro Lys Thr	
	345 350 355 360	
40	GTC ACC CAC AGT CTG AGG AAG ACC ATG AGG TTC CTG CAC AGC TTC ACA	1217
	Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His Ser Phe Thr	
	365 370 375	
	ATG TAC AGA CTG TAT CAG AAG CTC TTT TTA GAA ATG ATA GGG AAT CAG	1265
	Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn Gln	
	380 385 390	
45	GTT CAA TCC GTG AAA ATA AGC TGC TTA TAACTAGGAA TGGTCACTGG	1312
	Val Gln Ser Val Lys Ile Ser Cys Leu	
	395 400	
50	GCTGTTTCTT CA	1324

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(2) INFORMATION FOR SEQ ID NO:123:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 401 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

```

15 Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Leu Leu Asp Ile Ile
    1          5          10          15

    Glu Trp Thr Thr Gln Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp
        20          25          30

20 Pro Glu Thr Gly His Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
    35          40          45

    Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
    50          55          60

25 Cys Pro Asp His Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
    65          70          75          80

    Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu
    85          90          95

    Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
    100         105         110

35 Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser
    115         120         125

    Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys
    130         135         140

40 Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
    145         150         155         160

    Ile Lys His Thr Asn Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys
    165         170         175

    Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
    180         185         190

50 Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
    195         200         205

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Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val
 210 215 220
 5 Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
 225 230 235 240
 Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
 245 250 255
 10 Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln
 260 265 270
 15 Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Leu Gly His Ser
 275 280 285
 Asn Leu Thr Thr Glu Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly
 290 295 300
 20 Lys Lys Ile Ser Pro Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys
 305 310 315 320
 Ser Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
 325 330 335
 25 Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu
 340 345 350
 30 Lys Thr Ser His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr
 355 360 365
 Met Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu
 370 375 380
 35 Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
 385 390 395 400
 Leu

(2) INFORMATION FOR SEQ ID NO:124:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1355 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 94...1296

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

10	GTATATATAA CGTGATGAGC GTACGGGTGC GGAGACGCAC CGGAGCGCTC GCCCAGCCGC	60
	CGCTCCAAGC CCCTGAGGTT TCCGGGGACC ACA ATG AAC AAG TTG CTG TGC TGC	114
	Met Asn Lys Leu Leu Cys Cys	
	1 5	
15	GCG CTC GTG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG	162
	Ala Leu Val Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr	
	10 15 20	
20	TTT CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG	210
	Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu	
	25 30 35	
25	TTG TGT GAC AAA TGT CCT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA	258
	Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr	
	40 45 50 55	
30	GCA AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA	306
	Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr	
	60 65 70	
35	GAC AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC	354
	Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys	
	75 80 85	
40	AAG GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC	402
	Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg	
	90 95 100	
45	GTG TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG	450
	Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu	
	105 110 115	
50	AAA CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT GGA ACC	498
	Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr	
	120 125 130 135	
	CCA GAG CGA AAT ACA GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA	546
	Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser	
	140 145 150	

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	AAT GAG ACG TCA TCT AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser	594
	155 160 165	
5	GTC TTT GGT CTC CTG CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn	642
	170 175 180	
10	ATA TGT TCC GGA AAC AGT GAA TCA ACT CAA AAA TGT GGA ATA GAT GTT Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile Asp Val	690
	185 190 195	
15	ACC CTG TGT GAG GAG GCA TTC TTC AGG TTT GCT GTT CCT ACA AAG TTT Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Phe	738
	200 205 210 215	
20	ACG CCT AAC TGG CTT AGT GTC TTG GTA GAC AAT TTG CCT GGC ACC AAA Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly Thr Lys	786
	220 225 230	
25	GTA AAC GCA GAG AGT GTA GAG AGG ATA AAA CGG CAA CAC AGC TCA CAA Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser Ser Gln	834
	235 240 245	
30	GAA CAG ACT TTC CAG CTG CTG AAG TTA TGG AAA CAT CAA AAC AAA GCC Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Lys Ala	882
	250 255 260	
35	CAA GAT ATA GTC AAG AAG ATC ATC CAA GAT ATT GAC CTC TGT GAA AAC Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Asn	930
	265 270 275	
40	AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC TTC GAG CAG CTT Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu Gln Leu	978
	280 285 290 295	
45	CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG GGA GCA GAA GAC Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp	1026
	300 305 310	
50	ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC CAG ATC CTG AAG Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys	1074
	315 320 325	
55	CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA GAC ACC TTG AAG Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys	1122
	330 335 340	

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GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC CAC TTT CCC AAA 1170
 Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys
 345 350 355

5 ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC CTT CAC AGC TTC 1218
 Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe
 360 365 370 375

10 ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA ATG ATA GGT AAC 1266
 Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn
 380 385 390

CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA TAACTGGAAA TGGCCATTGA 1316
 Gln Val Gln Ser Val Lys Ile Ser Cys Leu
 15 395 400

GCTGTTTCCT CACAATTGGC GAGATCCCAT GGATGATAA 1355

20 (2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 401 amino acids
 (B) TYPE: amino acid
 25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

30 Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile
 1 5 10 15

35 Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
 20 25 30

Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr
 35 40 45

40 Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro
 50 55 60

Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
 65 70 75 80

45 Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu
 85 90 95

50 Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr
 100 105 110

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Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe
 115 120 125
 Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
 5 130 135 140
 Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys
 145 150 155 160
 10 Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys
 165 170 175
 Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr
 15 180 185 190
 Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
 195 200 205
 Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val
 20 210 215 220
 Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
 225 230 235 240
 25 Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
 245 250 255
 Trp Lys His Gln Asn Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln
 30 260 265 270
 Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala
 275 280 285
 35 Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly
 290 295 300
 Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys
 305 310 315 320
 40 Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
 325 330 335
 Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser
 45 340 345 350
 Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr
 355 360 365
 50 Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu
 370 375 380

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Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
 385 390 395 400

Leu

5

(2) INFORMATION FOR SEQ ID NO:126:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 139 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys
 1 5 10 15

25

Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
 20 25 30

30

Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala
 35 40 45

Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys
 50 55 60

35

Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr
 65 70 75 80

Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn
 85 90 95

40

Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His
 100 105 110

45

Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly
 115 120 125

Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys
 130 135

50

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(2) INFORMATION FOR SEQ ID NO:127:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

(2) INFORMATION FOR SEQ ID NO:128:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 219 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
 1 5 10 15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
 20 25 30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
 35 40 45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
 50 55 60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
 65 70 75 80

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[illegible]

(2) INFORMATION FOR SEQ ID NO:129:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

45 Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1 5 10 15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
20 25 30

50 His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
35 40 45

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Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
 50 55 60

5 Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
 65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
 85 90 95

10 Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
 100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
 115 120 125

15 Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
 130 135 140

20 Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
 145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
 165 170 175

25 Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
 180 185 190

30 Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
 195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
 210 215 220

35 Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
 225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
 245 250 255

40 Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
 260 265 270

45 Phe Ser Pro Thr Pro Gly Phe Thr
 275 280

50

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(2) INFORMATION FOR SEQ ID NO:130:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 207 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

15

Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly
 1 5 10 15

20

Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp
 20 25 30

Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr
 35 40 45

25

Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys
 50 55 60

Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val
 65 70 75 80

30

Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys
 85 90 95

Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys
 100 105 110

35

Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys
 115 120 125

Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr
 130 135 140

40

Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro
 145 150 155 160

45

Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn
 165 170 175

Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala Gly His Asn
 180 185 190

50

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Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr
 195 200 205

(2) INFORMATION FOR SEQ ID NO:131:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 227 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu
 1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr
 20 25 30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
 35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys
 50 55 60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp
 65 70 75 80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys
 85 90 95

Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg
 100 105 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu
 115 120 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg
 130 135 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val
 145 150 155 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr
 165 170 175

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Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly
 180 185 190
 5 Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser
 195 200 205
 Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser
 210 215 220
 10 Gln His Thr
 225

(2) INFORMATION FOR SEQ ID NO:132:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 197 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

30 Met Val Ser Leu Pro Arg Leu Cys Ala Leu Trp Gly Cys Leu Leu Thr
 1 5 10 15
 Ala Val His Leu Gly Gln Cys Val Thr Cys Ser Asp Lys Gln Tyr Leu
 20 25 30
 35 His Asp Gly Gln Cys Cys Asp Leu Cys Gln Pro Gly Ser Arg Leu Thr
 35 40 45
 Ser His Cys Thr Ala Leu Glu Lys Thr Gln Cys His Pro Cys Asp Ser
 50 55 60
 40 Gly Glu Phe Ser Ala Gln Trp Asn Arg Glu Ile Arg Cys His Gln His
 65 70 75 80
 Arg His Cys Glu Pro Asn Gln Gly Leu Arg Val Lys Lys Glu Gly Thr
 45 85 90 95
 Ala Glu Ser Asp Thr Val Cys Thr Cys Lys Glu Gly Gln His Cys Thr
 100 105 110
 50 Ser Lys Asp Cys Glu Ala Cys Ala Gln His Thr Pro Cys Ile Pro Gly
 115 120 125

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Phe Gly Val Met Glu Met Ala Thr Glu Thr Thr Asp Thr Val Cys His
 130 135 140
 5 Pro Cys Pro Val Gly Phe Phe Ser Asn Gln Ser Ser Leu Phe Glu Lys
 145 150 155 160
 Cys Tyr Pro Trp Thr Ser Cys Glu Asp Lys Asn Leu Glu Val Leu Gln
 165 170 175
 10 Lys Gly Thr Ser Gln Thr Asn Val Ile Cys Gly Leu Lys Ser Arg Met
 180 185 190
 Arg Ala Leu Leu Val
 15 195

(2) INFORMATION FOR SEQ ID NO:133:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 208 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile
 1 5 10 15
 35 Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
 20 25 30
 40 Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
 35 40 45
 Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
 50 55 60
 45 Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
 65 70 75 80
 Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu
 85 90 95
 50

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Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
 100 105 110
 Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu
 115 120 125
 Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
 130 135 140
 Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
 145 150 155 160
 Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln Lys
 165 170 175
 Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
 180 185 190
 Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
 195 200 205

(2) INFORMATION FOR SEQ ID NO:134:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 224 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

Met Gly Ala Gly Ala Thr Gly Arg Ala Met Asp Gly Pro Arg Leu Leu
 1 5 10 15
 Leu Leu Leu Leu Leu Gly Val Ser Leu Gly Gly Ala Lys Glu Ala Cys
 20 25 30
 Pro Thr Gly Leu Tyr Thr His Ser Gly Glu Cys Cys Lys Ala Cys Asn
 35 40 45
 Leu Gly Glu Gly Val Ala Gln Pro Cys Gly Ala Asn Gln Thr Val Cys
 50 55 60

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Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala Thr
 65 70 75 80
 Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met Ser
 5 85 90 95
 Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr Gly
 100 105 110
 Tyr Tyr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val Cys
 10 115 120 125
 Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln Asp Lys Gln Asn Thr
 130 135 140
 Val Cys Glu Glu Cys Pro Asp Gly Thr Tyr Ser Asp Glu Ala Asn His
 15 145 150 155 160
 Val Asp Pro Cys Leu Pro Cys Thr Val Cys Glu Asp Thr Glu Arg Gln
 20 165 170 175
 Leu Arg Glu Cys Thr Arg Trp Ala Asp Ala Glu Cys Glu Glu Ile Pro
 180 185 190
 Gly Arg Trp Ile Thr Arg Ser Thr Pro Pro Glu Gly Ser Asp Ser Thr
 25 195 200 205
 Ala Pro Ser Thr Gln Glu Pro Glu Ala Pro Pro Glu Gln Asp Leu Ile
 210 215 220
 30

(2) INFORMATION FOR SEQ ID NO:135:

(i) SEQUENCE CHARACTERISTICS:
 35 (A) LENGTH: 205 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

Met Tyr Val Trp Val Gln Gln Pro Thr Ala Phe Leu Leu Leu Gly Leu
 1 5 10 15
 Ser Leu Gly Val Thr Val Lys Leu Asn Cys Val Lys Asp Thr Tyr Pro
 20 25 30

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Ser Gly His Lys Cys Cys Arg Glu Cys Gln Pro Gly His Gly Met Val
 35 40 45
 5 Ser Arg Cys Asp His Thr Arg Asp Thr Val Cys His Pro Cys Glu Pro
 50 55 60
 Gly Phe Tyr Asn Glu Ala Val Asn Tyr Asp Thr Cys Lys Gln Cys Thr
 65 70 75 80
 10 Gln Cys Asn His Arg Ser Gly Ser Glu Leu Lys Gln Asn Cys Thr Pro
 85 90 95
 Thr Glu Asp Thr Val Cys Gln Cys Arg Pro Gly Thr Gln Pro Arg Gln
 100 105 110
 15 Asp Ser Ser His Lys Leu Gly Val Asp Cys Val Pro Cys Pro Pro Gly
 115 120 125
 His Phe Ser Pro Gly Ser Asn Gln Ala Cys Lys Pro Trp Thr Asn Cys
 130 135 140
 Thr Leu Ser Gly Lys Gln Ile Arg His Pro Ala Ser Asn Ser Leu Asp
 145 150 155 160
 25 Thr Val Cys Glu Asp Arg Ser Leu Leu Ala Thr Leu Leu Trp Glu Thr
 165 170 175
 Gln Arg Thr Thr Phe Arg Pro Thr Thr Val Pro Ser Thr Thr Val Trp
 180 185 190
 30 Pro Arg Thr Ser Gln Leu Pro Ser Thr Pro Thr Leu Val
 195 200 205

35 (2) INFORMATION FOR SEQ ID NO:136:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
 (B) TYPE: amino acid
 40 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

50 Met Gly Asn Asn Cys Tyr Asn Val Val Val Ile Val Leu Leu Leu Val
 1 5 10 15

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Gly Cys Glu Lys Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln
 20 25 30
 5 Pro Gly Thr Phe Cys Arg Lys Tyr Asn Pro Val Cys Lys Ser Cys Pro
 35 40 45
 Pro Ser Thr Phe Ser Ser Ile Gly Gly Gln Pro Asn Cys Asn Ile Cys
 50 55 60
 10 Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Lys Phe Cys Ser Ser Thr
 65 70 75 80
 His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro
 85 90 95
 15 Gln Cys Thr Arg Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr
 100 105 110
 20 Lys Gln Gly Cys Lys Thr Cys Ser Leu Gly Thr Phe Asn Asp Gln Asn
 115 120 125
 Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser Leu Asp Gly Arg
 130 135 140
 25 Ser Val Leu Lys Thr Gly Thr Thr Glu Lys Asp Val Val Cys Gly Pro
 145 150 155 160
 Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu
 165 170 175
 30 Gly Gly Pro Gly Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu
 180 185 190

35 (2) INFORMATION FOR SEQ ID NO:137:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTAC

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(2) INFORMATION FOR SEQ ID NO:138:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 380 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

15 Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His
 1 5 10 15

20 Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His
 20 25 30

Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser
 35 40 45

25 Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro
 50 55 60

30 Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His
 65 70 75 80

Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe
 85 90 95

35 Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala
 100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe
 115 120 125

40 Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn
 130 135 140

Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys Gly Asn Ala Thr His
 145 150 155 160

45 Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile
 165 170 175

50 Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr
 180 185 190

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Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly
 195 200 205
 5 Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser
 210 215 220
 Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn
 225 230 235 240
 10 Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys
 245 250 255
 Glu Ser Ser Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu
 260 265 270
 15 Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro
 275 280 285
 Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu
 290 295 300
 20 Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr
 305 310 315 320
 25 Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe
 325 330 335
 Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His
 340 345 350
 30 Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile
 355 360 365
 35 Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
 370 375 380

(2) INFORMATION FOR SEQ ID NO:139:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 380 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 45 (ii) MOLECULE TYPE: protein
 50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

5	Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His	1 5 10 15
10	Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His	20 25 30
15	Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr	35 40 45
20	Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro	50 55 60
25	Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His	65 70 75 80
30	Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe	85 90 95
35	Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala	100 105 110
40	Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe	115 120 125
45	Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn	130 135 140
50	Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His	145 150 155 160
55	Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile	165 170 175
60	Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr	180 185 190
65	Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly	195 200 205
70	Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser	210 215 220
75	Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn	225 230 235 240
80	Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys	245 250 255

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Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu
 260 265 270
 5 Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala
 275 280 285
 Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile
 290 295 300
 10 Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr
 305 310 315 320
 Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe
 325 330 335
 15 Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His
 340 345 350
 20 Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile
 355 360 365
 Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
 370 375 380

25 (2) INFORMATION FOR SEQ ID NO:140:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 30 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

40 TGGACCACCC AGAAGTACCT TCATTATGAC

30

(2) INFORMATION FOR SEQ ID NO:141:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 45 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

GTCATAATGA AGGTACTTCT GGGTGGTCCA

30

10 (2) INFORMATION FOR SEQ ID NO:142:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

25 GGACCACCCA GCTTCATTAT GACGAAGAAA C

31

(2) INFORMATION FOR SEQ ID NO:143:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

GTTTCTTCGT CATAATGAAG CTGGGTGGTC C

31

(2) INFORMATION FOR SEQ ID NO:144:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

50 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GTGGACCACC CAGGACGAAG AAACCTCTC

29

10 (2) INFORMATION FOR SEQ ID NO:145:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GAGAGGTTTC TTCGTCCTGG GTGGTCCAC

29

25

(2) INFORMATION FOR SEQ ID NO:146:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:

40

CGTTTCCTCC AAAGTTCCTT CATTATGAC

29

(2) INFORMATION FOR SEQ ID NO:147:

45 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:

5

GTCATAATGA AGGAACTTTG GAGGAAACG

29

(2) INFORMATION FOR SEQ ID NO:148:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

GGAAACGTTT CCTGCAAAGT ACCTTCATTA TG

32

25

(2) INFORMATION FOR SEQ ID NO:149:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

30

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

40

CATAATGAAG GTACTTTGCA GGAAACGTTT CC

32

(2) INFORMATION FOR SEQ ID NO:150:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

45

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

- 222 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

5 CACGCAAAAG TCGGGAATAG ATGTCAC

27

(2) INFORMATION FOR SEQ ID NO:151:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

GTGACATCTA TTCCCGACTT TTGCGTG

27

(2) INFORMATION FOR SEQ ID NO:152:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

40 CACCCTGTCG GAAGAGGCCT TCTTC

25

(2) INFORMATION FOR SEQ ID NO:153:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GAAGAAGGCC TCTTCCGACA GGGTG

25

(2) INFORMATION FOR SEQ ID NO:154:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

TGACCTCTCG GAAAGCAGCG TGCA

24

25

(2) INFORMATION FOR SEQ ID NO:155:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:

40

TGCACGCTGC TTTCCGAGAG GTCA

24

(2) INFORMATION FOR SEQ ID NO:156:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

- 224 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:

5

CCTCGAAATC GAGCGAGCAG CTCC

24

(2) INFORMATION FOR SEQ ID NO:157:

10

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:

CGATTCGAG GTCTTCTCG TTCTC

25

25

(2) INFORMATION FOR SEQ ID NO:158:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

40

CCGTGAAAAT AAGCTCGTTA TAACTAGGAA TGG

33

(2) INFORMATION FOR SEQ ID NO:159:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

45

50

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

5 CCATTCCTAG TTATAACGAG CTTATTTTCA CGG 33

(2) INFORMATION FOR SEQ ID NO:160:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG 38

(2) INFORMATION FOR SEQ ID NO:161:

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:

CCTCTCTCGA GTCAGGTGAC ATCTATTCCA CACTTTTGCG TGGC 44

40

(2) INFORMATION FOR SEQ ID NO:162:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
45 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

10 (2) INFORMATION FOR SEQ ID NO:163:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:

25 CCTCTCTCGA GTCAAGGAAC AGCAAACCTG AAGAAGGC

38

(2) INFORMATION FOR SEQ ID NO:164:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:

40

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:165:

45 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 227 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:

5 CCTCTCTCGA GTCACTCTGT GGTGAGGTTT GAGTGGCC 38

(2) INFORMATION FOR SEQ ID NO:166:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG 38

(2) INFORMATION FOR SEQ ID NO:167:

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

40 CCTCTCTCGA GTCAGGATGT TTTCAAGTGC TTGAGGGC 38

(2) INFORMATION FOR SEQ ID NO:168:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

- 228 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:168:

Met	Lys	His	His	His	His	His	His	His	Ala	Ser	Val	Asn	Ala	Leu	Glu
1				5					10					15	

(Ref No-12/95 Pub No15)

FORM 13-27

13-205

Applicant's or agent's file reference number	A-378-CIP2	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>15, 16, 45, 60, 64, 67, 100</u> line <u>too many to list</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection (ATCC)	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, MD 20852	
Date of deposit 12/27/95 and 7/24/96	Accession Number 69969, 69970, 69971, 98112 and 98113
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer Enora Rivera Paralegal Specialist IAPD - PCT Operations (703) 365-6676	Authorized officer

- 230 -

WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding a
5 polypeptide comprising at least one of the biological
activities of OPG wherein the nucleic acid is selected
from the group consisting of:
 - a) the nucleic acids shown in Figures 2B-
2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D
10 (SEQ ID NO:124) or complementary strands thereof;
 - b) nucleic acids which hybridize under
stringent conditions with the polypeptide-encoding
regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B
(SEQ ID NO:122) and 9C-9D (SEQ ID NO:124);
 - 15 c) nucleic acids which hybridize under
stringent conditions with nucleotides 148 through 337
inclusive as shown in Figure 1A; and
 - d) nucleic acid which are degenerate to
the nucleic acids of (a), (b) and (c).
- 20 2. The nucleic acid of Claim 1 which is cDNA,
genomic DNA, synthetic DNA or RNA.
3. A polypeptide encoded by the nucleic acid
25 of Claim 1.
4. The nucleic acid of Claim 1 including one
or more codons preferred for Escherichia coli
expression.
- 30 5. The nucleic acid of Claim 1 having a
detectable label attached thereto.

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6. The nucleic acid of Claim 1 comprising the polypeptide-encoding region of Figure 2B-2C (SEQ ID NO:120), Figure 9A-9B (SEQ ID NO:122) or Figure 9C-9D (SEQ ID NO:124).

5

7. The nucleic acid of Claim 6 having the sequence as shown in Figure 9C-D (SEQ ID NO:124) from nucleotides 158-1297.

10

8. An expression vector comprising the nucleic acid of Claim 1.

15

9. The expression vector of Claim 8 wherein the nucleic acid comprises the polypeptide - encoding region as shown in Figure 9C-9D (SEQ ID NO:124).

10. A host cell transformed or transfected with the expression vector of Claim 8.

20

11. The host cell of Claim 10 which is a eucaryotic cell.

25

12. The host cell of Claim 11 which is selected from the group consisting of CHO, COS, 293, 3T3, CV-1 and BHK cells.

13. The host cell of Claim 10 which is a procaryotic cell.

30

14. The host cell of Claim 13 which is Escherichia coli.

15. A transgenic mammal comprising the expression vector of Claim 8.

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16. The transgenic mammal of Claim 15 which is a rodent.

5 17. The transgenic mammal of Claim 16 which is a mouse.

18. A process for the production of OPG comprising:
10 growing under suitable nutrient conditions host cells transformed or transfected with the nucleic acid of Claim 1; and
isolating the polypeptide products of the expression of the nucleic acids.

15 19. A purified and isolated polypeptide comprising OPG.

20 20. The polypeptide of Claim 19 which is mammalian OPG.

21. The polypeptide of Claim 20 which is human OPG.

25 22. The polypeptide of Claim 19 which is substantially free of other human proteins.

30 23. The polypeptide of Claim 21 having the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123), or Figure 9C-9D (SEQ ID NO:125) or a derivative thereof.

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24. The polypeptide of Claim 23 having the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-401 inclusive.

5 25. The polypeptide of Claim 23 having the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 32-401 inclusive.

10 26. The polypeptide of Claim 19 which is characterized by being a product of expression of an exogenous DNA sequence.

15 27. The polypeptide of Claim 26 wherein the DNA is cDNA, genomic DNA or synthetic DNA.

 28. The polypeptide of Claim 19 which has been modified with a water-soluble polymer.

20 29. The polypeptide of Claim 28 wherein the water soluble polymer is polyethylene glycol.

 30. A polypeptide comprising:
 an amino acid sequence of at least about 164 amino acids comprising four cysteine-rich domains
25 characteristic of the cysteine rich domains of tumor necrosis factor receptor extracellular regions; and
 an activity of increasing bone density.

30 31. A polypeptide comprising the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID NO:125) having an amino terminus at residue 22, and wherein from 1 to 216 amino acids are deleted from the carboxy terminus.

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32. The polypeptide of Claim 31 comprising the amino acid sequence from residues 22-185, 22-189, 22-194, or 22-201 inclusive.

5

33. The polypeptide of Claim 32 further comprising an Fc region of human IgG1 extending from the carboxy terminus.

10

34. A polypeptide comprising the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID NO:125) having an amino terminus at residue 22, wherein from 1 to 10 amino acids are deleted from the amino terminus and, optionally, from 1 to 216 amino acids are deleted from the carboxy terminus.

15

35. The polypeptide of Claim 34 comprising the amino acid sequence from residues 27-185, 27-189, 27-194, 27-401, or 32-401 inclusive.

20

36. The polypeptide of Claim 35 further comprising an Fc region of human IgG1 extending from the carboxy terminus.

25

37. A polypeptide selected from the group consisting of:

30

huOPG [22-201]-Fc
huOPG [22-401]-Fc
huOPG [22-180]-Fc
huOPG met [22-401]-Fc
huOPG Fc-met [22-401]
huOPG met [22-185]
huOPG met [22-189]

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5 huOPG met [22-194]
huOPG met [27-185]
huOPG met [27-189]
huOPG met [27-194]
huOPG met [32-401]
huOPG met-lys[22-401]
huOPG met [22-401]
huOPG met [22-401]-Fc (P25A)
huOPG met [22-401] (P25A)
10 huOPG met [22-401] (P26A)
huOPG met [22-401] (P26D)
huOPG met [22-194] (P25A)
huOPG met [22-194] (P26A)
huOPG met met-(lys)₃ [22-401]
15 huOPG met met-arg-gly-ser-(his)₆ [22-401]

38. A nucleic acid encoding the polypeptide of Claim 37.

20 39. An antibody or fragment thereof which specifically binds to OPG.

40. The antibody of Claim 39 which is a monoclonal antibody.

25

41. A method for detecting the presence of OPG in a biological sample comprising:

incubating the sample with the antibody of Claim 39 under conditions that allow binding of the
30 antibody to OPG; and

detecting the bound antibody.

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42. A method to assess the ability of a candidate substance to bind to OPG comprising:
incubating OPG with the candidate substance
5 under conditions that allow binding; and
measuring the bound substance.
43. A method of regulating the levels of OPG in an animal comprising modifying the animal with a
10 nucleic acid encoding OPG.
44. The method of Claim 43 wherein the nucleic acid promotes an increase in the tissue level of OPG.
15
45. The method of Claim 44 wherein the animal is a human.
46. A pharmaceutical composition comprising a
20 therapeutically effective amount of OPG in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.
47. The composition of Claim 46 wherein the
25 OPG is human OPG.
48. The composition of Claim 47 wherein the OPG has the amino acid sequence as shown in Figure 9B.
49. A method of treating a bone disorder comprising administering a therapeutically effective amount of the polypeptide of Claim 19.
30

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50. The method of Claim 49 wherein the polypeptide is human OPG.

51. The method of Claim 49 wherein the bone disorder is excessive bone loss.

52. The method of Claim 51 wherein the bone disorder is selected from the group consisting of osteoporosis, Paget's disease of bone, hypercalcemia, hyperparathyroidism, steroid-induced osteopenia, bone loss due to rheumatoid arthritis, bone loss due to osteomyelitis, osteolytic metastasis, and periodontal bone loss.

53. The method of Claim 49 further comprising administering a therapeutically effective amount of a substances selected from the group consisting of bone morphogenic proteins BMP-1 through BMP-12, TGF- β family members, IL-1 inhibitors, TNF α inhibitors, parathyroid hormone and analogs thereof, parathyroid hormone related protein and analogs thereof, E series prostaglandins, bisphosphonates, and bone-enhancing minerals.

54. An osteoprotegerin multimer consisting of osteoprotegerin monomers.

55. The multimer of Claim 54 which is a dimer.

56. The multimer of Claim 54 formed by interchain disulfide bonds.

57. The multimer of Claim 54 formed by association Fc regions derived from human IgG1.

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58. The multimer of Claim 54 which is essentially free of osteoprotegerin monomers and inactive multimers.

5

59. The multimer of Claim 54 wherein the monomers comprise the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-401, or a derivative thereof.

10

60. The multimer of Claim 54 wherein the monomers comprise the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-194.

FIG. 1A

148	178	208	238	268	298					
FRI-1	ALLVFLDII	IEWTTQET	FFPKYLHYD	PETGRQLLCD	KCAPGTYLKQHCTVRRKTL	LCVPCPD				
SW:TNR2_HUMAN	HALPAQVAFT	PYAPEGSTC	RLREYYDQ	TAQMCCSKC	SPGQHA	KVFC	TKTSD	TVCD	SCED	
	30	40	50	60	70	80				
328	YSYTD	SWHTS								
FRI-1	: : :									
SW:TNR2_HUMAN	STYTQLWN	WVPECL	SCGSR	CSSDQ	VETQACT	TREQNR	ICTCR	PGWY	CALSKQEG	RCLCAPL
	90	100	110	120	130	140				

FIG. 1B

FRI-1	69	YLHYDPETGRQLLCDKCAPGTYLKQHC.TVRRKTLCV.PCPDY.SYTDSW
	 : :
TNFR profile	6	YHYDQNGRMCEECHMCQPGHFLVKHCKQPKRDTVCHKPCPEPGVTYTTDDW
FRI-1	116	H
TNFR profile	56	H

Z Score = 8.29

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FIG. 1C

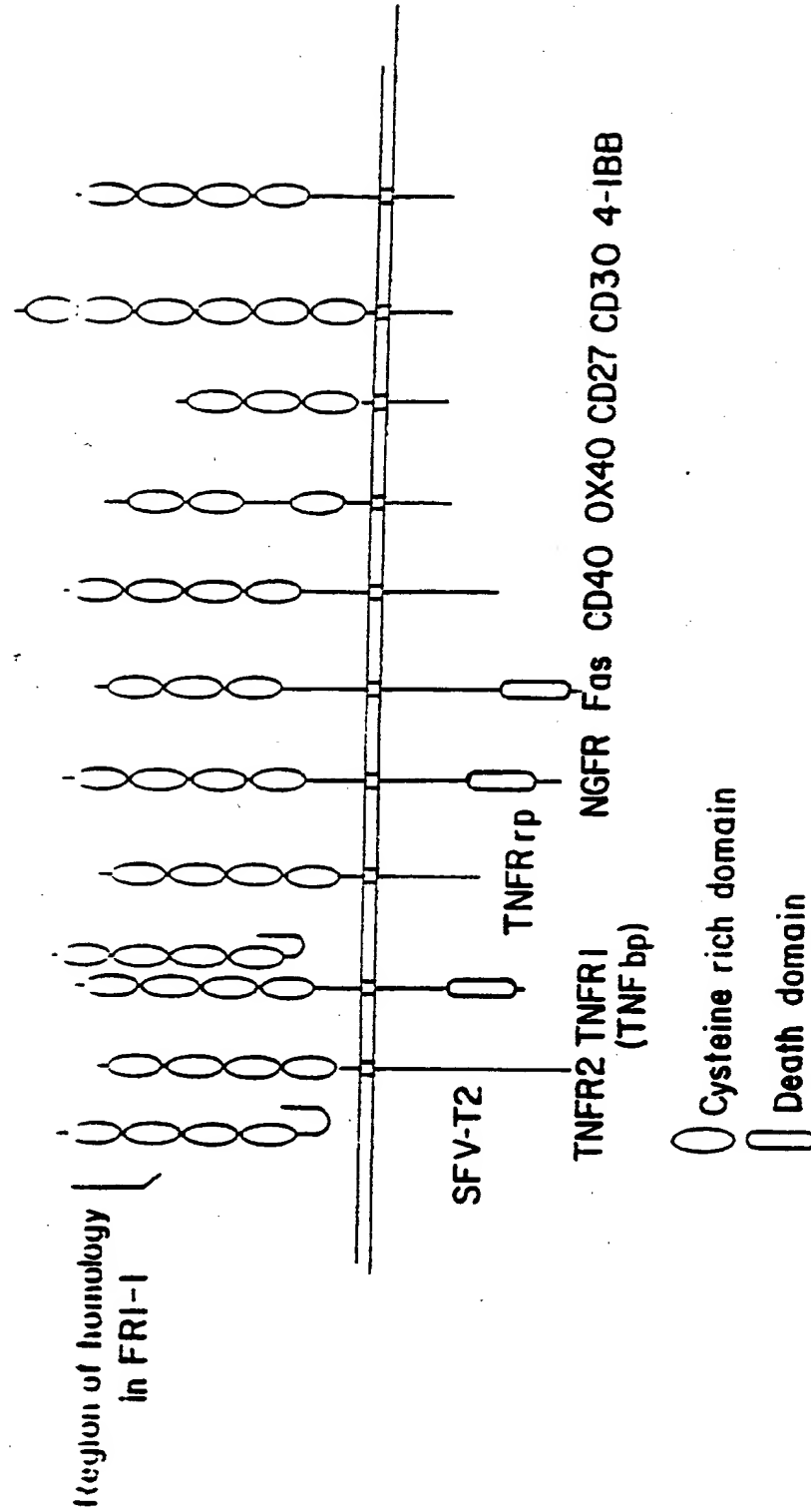


FIG.2A

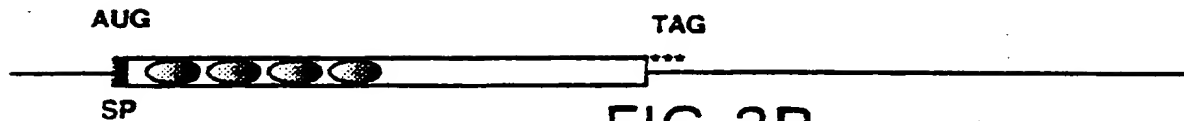


FIG.2B

10 30 50
 ATCAAAGGCAGGGCATACTTCCTGTTGCCAGACCTTATATAAAACGTCATGTTTCGCCTG
 70 90 110
 GGCAGCAGAGAAGCACCTAGCACTGGCCCAGCGGCTGCCGCCTGAGGTTTCCAGAGGACC
 130 150 170
 ACAATGAACAAGTGGCTGTGCTGTGCACTCCTGGTGTCTTGGACATCATTGAATGGACA
 M N K W L C C A L L V F L D I I E W T
 190 210 230
 ACCCAGGAAACCTTTCTCTCCAAAATACTTGCATTATGACCCAGAAACCGGACGTCAGCTC
 T Q E T F P P K Y L H Y D P E T G R Q L
 250 270 290
 TTGTGTGACAAATGTGCTCCTGGCACCTACCTAAACAGCACTGCACAGTCAGGAGGAAG
 L C D K C A P G T Y L K Q H C T V R R K
 310 330 350
 ACACTGTGTGTCCCTTGCCCTGACTACTCTTATACAGACAGCTGGCACACGAGTGATGAA
 T L C V P C P D Y S Y T D S W H T S D E
 370 390 410
 TGCGTGTACTGCAGCCCCGTGTGCAAGGAACTGCAGACCGTGAAACAGGAGTGCAACCGC
 C V Y C S P V C K E L Q T V K Q E C N R
 430 450 470
 ACCCACAACCGAGTGTGCGAATGTGAGGAAGGGCGCTACCTGGAGCTCGAATTCTGCTTG
 T H N R V C E C E E G R Y L E L E F C L
 490 510 530
 AAGCACCGGAGCTGTCCCCCAGGCTTGGGTGTGCTGCAGGCTGGGACCCAGAGCGAAAC
 K H R S C P P G L G V L Q A G T P E R N
 550 570 590
 ACGGTTTGCAAAAGATGTCCGGATGGGTCTTCTCAGGTGAGACGTCATCGAAAGCACCC
 T V C K R C P D G F F S G E T S S K A P
 610 630 650
 TGTAGGAAACACACCAACTGCAGCTCACTTGGCCTCCTGCTAATTCAGAAAGGAAATGCA
 C R K H T M C S S L G L L L I Q K G N A
 670 690 710
 ACACATGACAATGTATGTTCCGGAAACAGAGAAGCAACTCAAAATTGTGGAATAGATGTC
 T H D N V C S G N R E A T Q N C G I D V
 730 750 770
 ACCCTGTGCGAAGAGGCATTCTTCAGGTTTGTGCTGTGCCTACCAAGATTATACCGAATTGG
 T L C E E A F F R F A V P T K I I P N W
 790 810 830
 CTGAGTGTCTGCTGGACAGTTTGCCTGGGACCAAAGTGAATGCAGAGAGTGTAGAGAGG
 L S V L V D S L P G T K V N A E S V E R
 850 870 890
 ATAAAACGGAGACACAGCTCGCAAGAGCAAACCTTCCAGCTACTTAAGCTGTGGAAGCAT
 I K R R H S S Q E Q T F Q L L K L W K H
 910 930 950
 CAAAACAGAGACCAGGAAATGGTGAAGAAGATCATCCAAGACATTGACCTCTGTGAAAGC
 Q N R D Q E M V K K I I Q D I D L C E S
 970 990 1010
 AGTGTGCAACGGCATATCGGCCACGCGAACCTCACCACAGAGCAGCTCCGCATCTTGATG
 S V Q R H I G H A N L T T E Q L R I L M

FIG.2C

1030 1050 1070
GAGAGCTTGCCTGGGAAGAAGATCAGCCCAGACGAGATTGAGAGAACGAGAAAGACCTGC
E S L P G K K I S P D E I E R T R K T C
1090 1110 1130
AAACCCAGCGAGCAGCTCCTGAAGCTACTGAGCTTGTGGAGGATCAAAAATGGAGACCAA
K P S E Q L L K L L S L W R I K N G D Q
1150 1170 1190
GACACCTTGAAGGGCCTGATGTACGCACTCAAGCACTTGAAAGCATACCCTTTCCCAA
D T L K G L M Y A L K H L K A Y H F P K
1210 1230 1250
ACCGTCACCCACAGTCTGAGGAAGACCATCAGGTTCTTGACAGCTTCACCATGTACCGA
T V T H S L R K T I R F L H S F T M Y R
1270 1290 1310
TTGTATCAGAACTCTTTCTAGAAATGATAGGGAATCAGGTTCAATCAGTGAAGATAAGC
L Y Q K L F L E M I G N Q V Q S V K I S
1330 1350 1370
TGCTTATAGTTAGGAATGGTCACTGGGCTGTTTCTTCAGGATGGGCCAACACTGATGGAG
C L
1390 1410 1430
CAGATGGCTGCTTCTCCGGCTCTTGAAATGGCAGTTGATTCCTTTCTCATCAGTTGGTGG
1450 1470 1490
GAATGAAGATCCTCCAGCCCAACACACACTGGGGAGTCTGAGTCAGGAGAGTGAGGCA
1510 1530 1550
GGCTATTTGATAATTGTGCAAAGCTGCCAGGTGTACACCTAGAAAGTCAAGCACCTGAG
1570 1590 1610
AAAGAGGATATTTTTATAACCTCAAACATAGGCCCTTTCCTTCCTCTCCTTATGGATGAG
1630 1650 1670
TACTCAGAAGGCTTCTACTATCTTCTGTGTCATCCCTAGATGAAGGCCTCTTTTATTTAT
1690 1710 1730
TTTTTTATTCTTTTTTTCGGAGCTGGGGACCGAACCCAGGGCCTTGCGCTTGCGAGGCAA
1750 1770 1790
GTGCTCTACCACTGAGCTAAATCTCCAACCCCTGAAGGCCTCTTTCTTTCTGCCTCTGAT
1810 1830 1850
AGTCTATGACATTCTTTTTTCTACAATTTCGTATCAGGTGCACGAGCCTTATCCCATTGT
1870 1890 1910
AGGTTTCTAGGCAAGTTGACCGTTAGCTATTTTCCCTCTGAAGATTTGATTTCGAGTTGC
1930 1950 1970
AGACTTGGCTAGACAAGCAGGGGTAGGTTATGGTAGTTTATTTAACAGACTGCCACCAGG
1990 2010 2030
AGTCCAGTGTTTCTTGTTCTCTGTAGTTGTACCTAAGCTGACTCCAAGTACATTTAGTA
2050 2070 2090
TGAAAAATAATCAACAAATTTTATTCCTTCTATCAACATTGGCTAGCTTTGTTTCAGGGC
2110 2130 2150
ACTAAAAGAACTACTATATGGAGAAAGAATTGATATTGCCCCCAACGTTCAACAACCCA
2170 2190 2210
ATAGTTTATCCAGCTGTCATGCCTGGTTTCAGTGTCTACTGACTATGCGCCCTCTTATTAC
2230 2250 2270
TGCATGCAGTAATTCAACTGGAAATAGTAATAATAATAAGAAATAAAATCTAGACTCC
2290 2310 2330
ATTGGATCTCTCTGAATATGGGAATATCTAACTTAAGAAGCTTTGAGATTTTCAGTTGTGT
2350 2370 2390
TAAAGGCTTTTATTAAAAAGCTGATGCTCTTCTGTAAAGTTACTAATATATCTGTAAGA
2410 2430
CTATTACAGTATTGCTATTTATATCCATCCAG

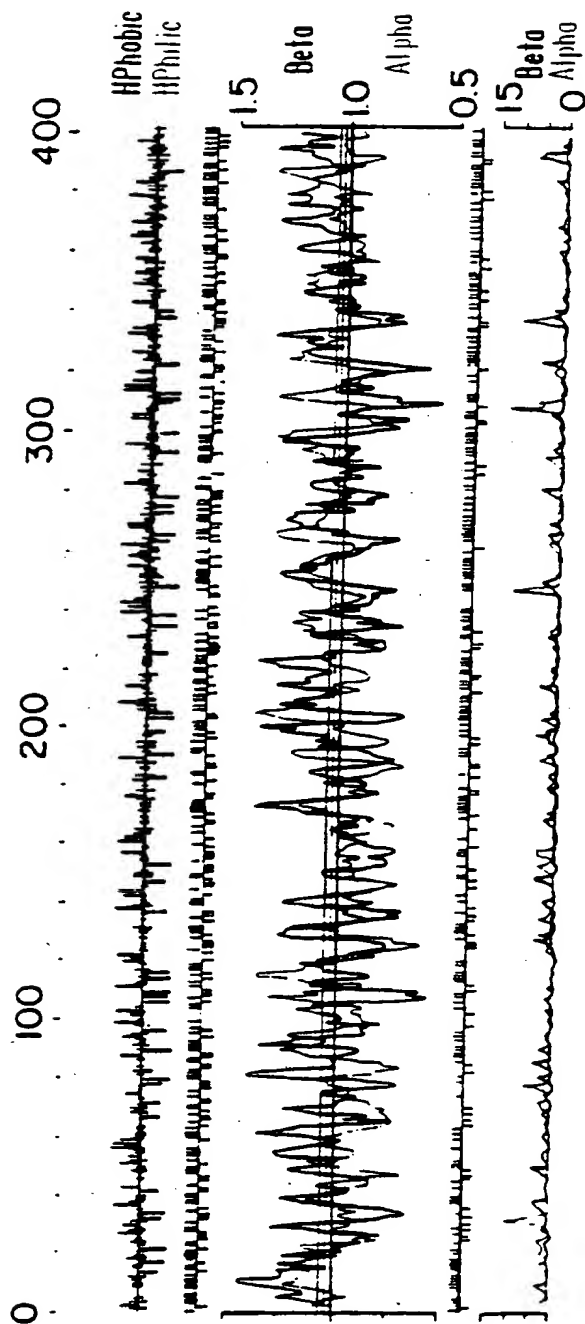


FIG. 3A

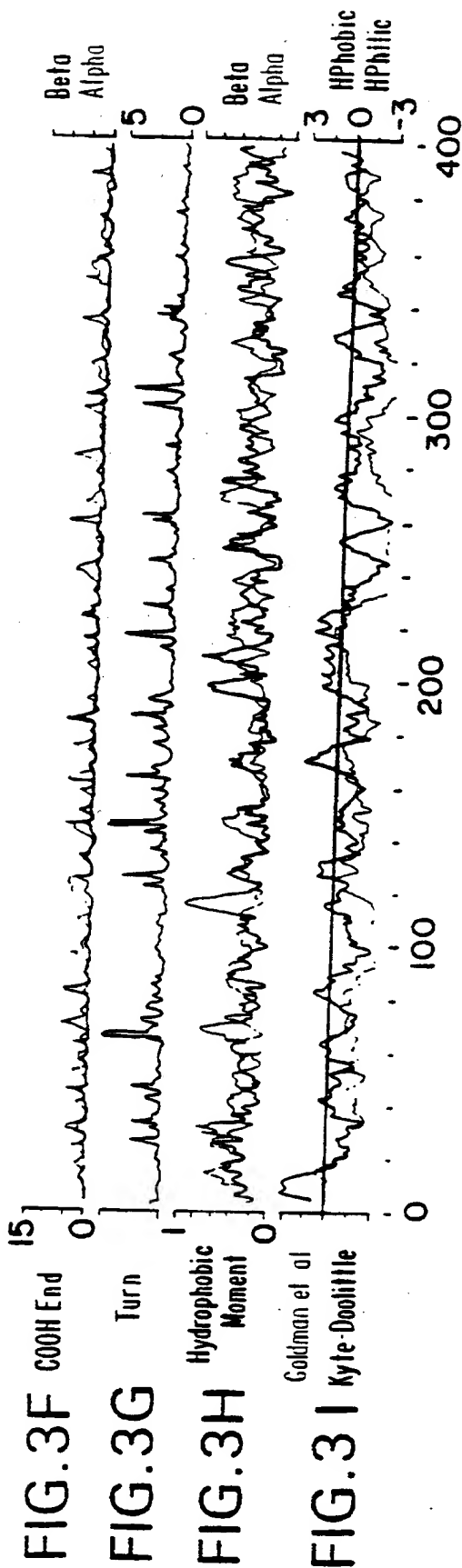
FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

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FIG. 4A

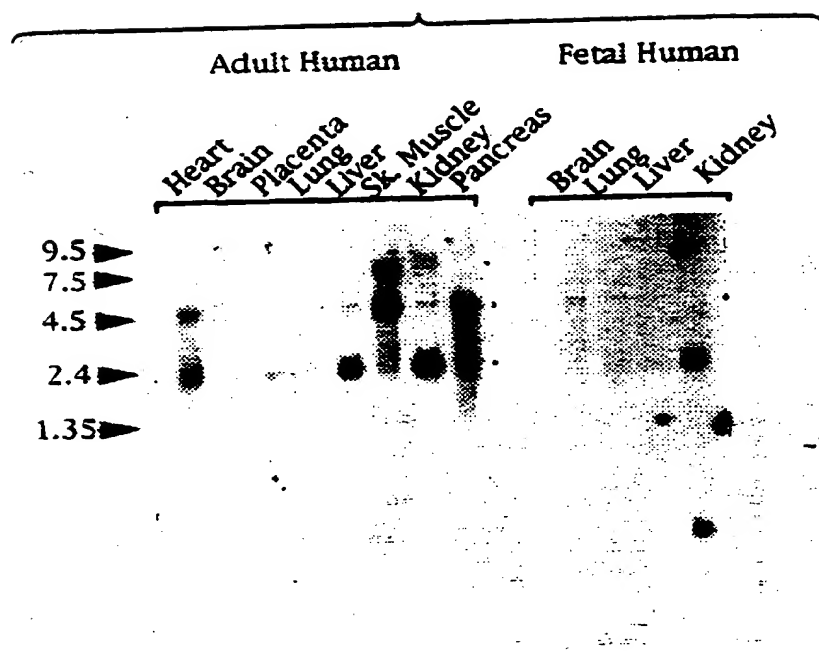
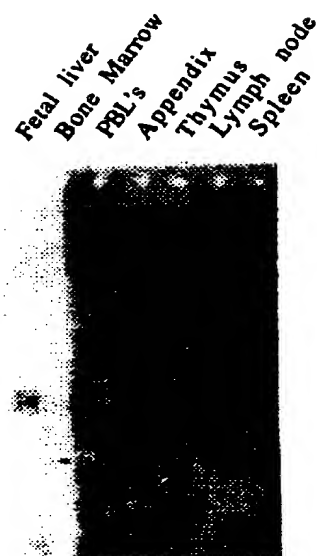


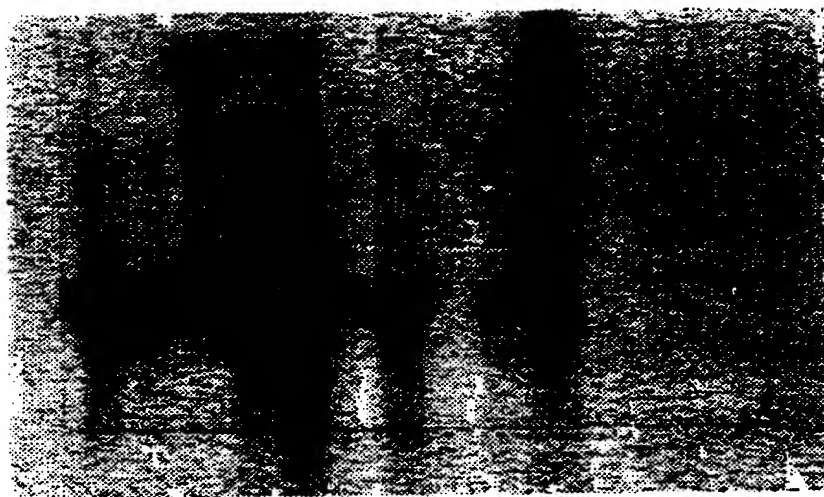
FIG. 4B

Human Immune System



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FIG.5



2 11 16 17 22 28 33 38 45 Kb 1 12 18 30

Transgenic Founders

Controls

FIG. 6A

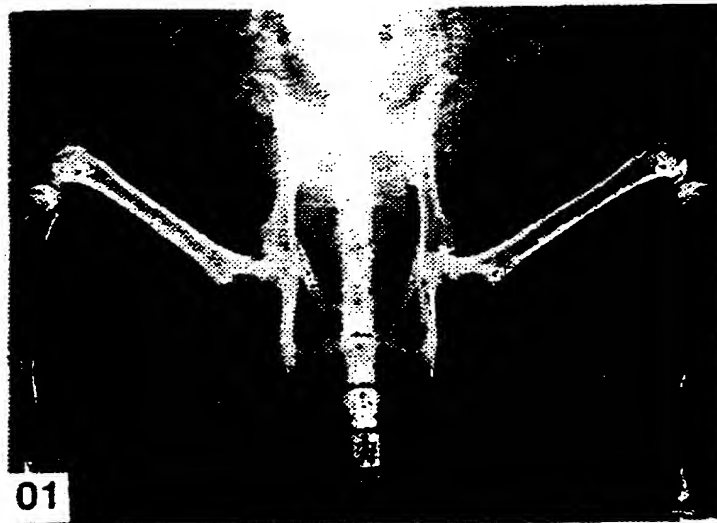


FIG. 6B

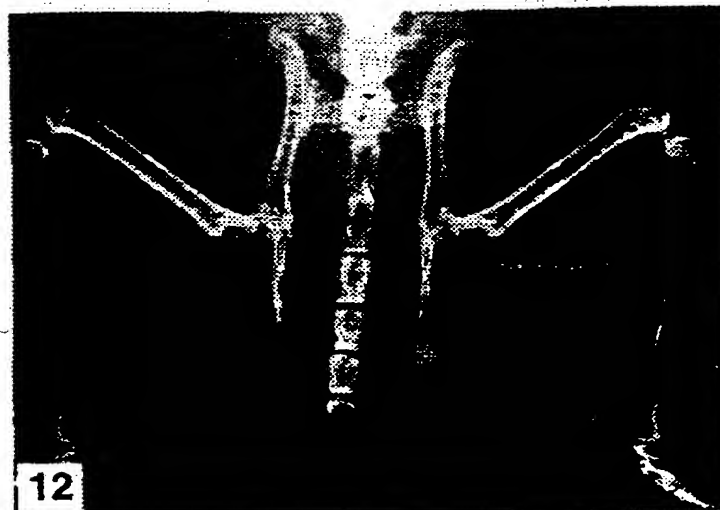


FIG. 6C

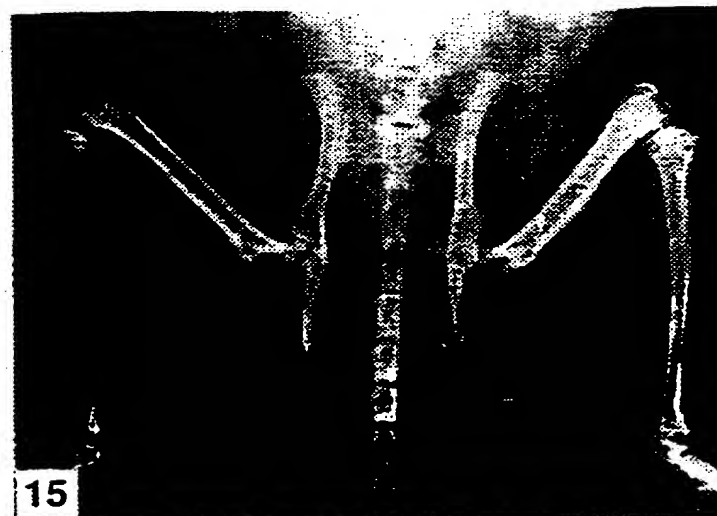


FIG. 6D

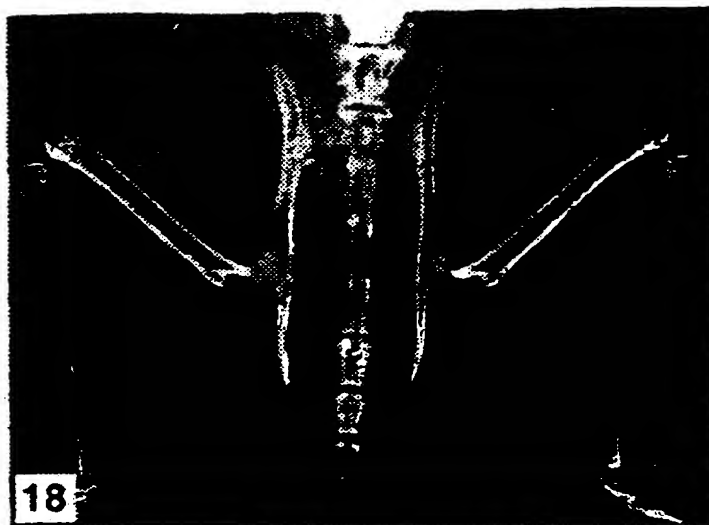
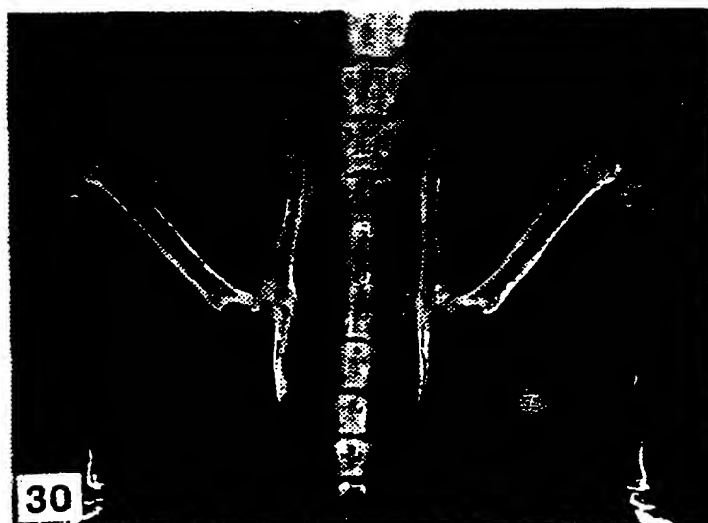


FIG. 6E



FIG. 6F



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FIG. 6G

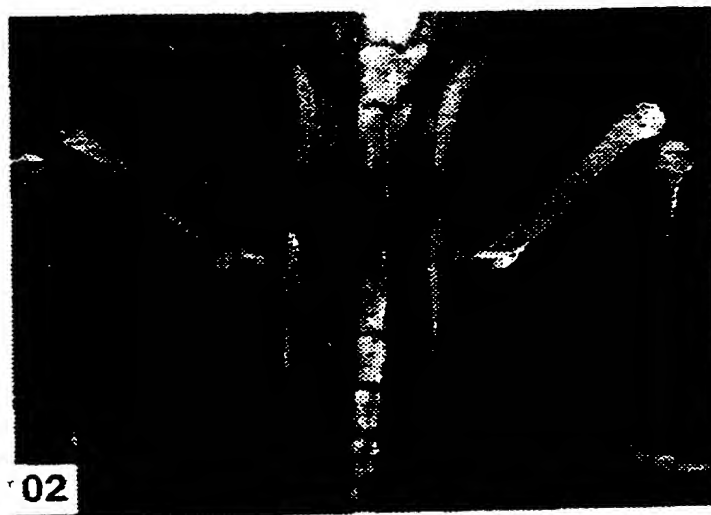
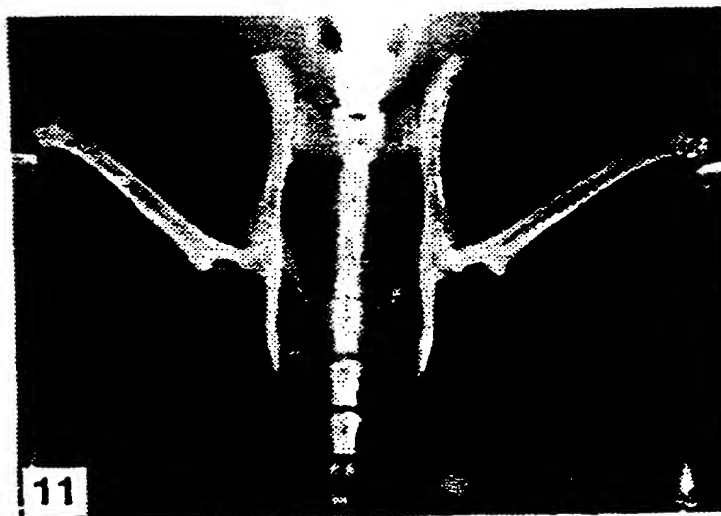


FIG. 6H



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FIG. 9A

10 30 50
CCTTATATAARACGTCATGATTGCCTGGGCTGCAGAGACGCACCTAGCACTGACCCAGCG
70 90 110
GCTGCCTCCTGAGGTTTCCCGAGGACCACAATGAACAAGTGGCTGTGCTGCGCACTCCTG
130 150 170
GTGCTCCTGGACATCATTGAATGGACAACCCAGGAAACCCCTTCCTCCAAAGTACTTGCAT
V L L D I I E W T T O E T L P P K Y L H
190 210 230
TATGACCCAGAACTGGTCATCAGCTCCTGTGTGACAAATGTGCTCCTGGCACCTACCTA
Y D P E T G H Q L L C D K C A P G T Y L
250 270 290
AAACAGCACTGCACAGTGAGGAGGAAGACATTGTGTGTCCCTTGCCCTGACCCTCTTAT
K Q H C T V R R K T L C V P C P D H S Y
310 330 350
ACGGACAGCTGGCACACCAGTGATGAGTGTGTGTATTGCAGCCCAGTGTGCAAGGAACTG
T D S W H T S D E C V Y C S P V C K E L
370 390 410
CAGTCCGTGAAGCAGGAGTGCAACCGCACCCACAACCGAGTGTGTGAGTGTGAGGAAGGG
Q S V K Q E C N R T H N R V C E C E E G
430 450 470
CGTTACCTGGAGATCGAATTCTGCTTGAAGCACCGGAGCTGTCCCCCGGGCTCCGGCGTG
R Y L E I E F C L K H R S C P P G S G V
490 510 530
GTGCAAGCTGGAACCCAGAGCGAAACACAGTTTGCAAAAAATGTCCAGATGGGTTCTTC
V Q A G T P E R N T V C K K C P D G F F
550 570 590
TCAGGTGAGACTTCATCGAAAGCACCCCTGTATAAAACACACGAACTGCAGCACATTTGGC
S G E T S S K A P C I K H T N C S T F G
610 630 650
CTCCTGCTAATTCAGAAAGGAAATGCAACACATGACAACGTGTGTTCCGGAAACAGAGAA
L L L I Q K G N A T H D N V C S G N R E
670 690 710
GCCACGCAAAAGTGTGGAATAGATGTCACCCTGTGTGAAGAGGCCTTCTTCAGGTTTGCT
A T Q K C G I D V T L C E E A F F R F A
730 750 770
GTTCTACCAAGATTATACCAAATTGGCTGAGTGTGTTTGGTGGACAGTTTGCCTGGGACC
V P T K I I P N W L S V L V D S L P G T

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FIG. 9B

790 810 830
AAAGTGAATGCCGAGAGTGTAGAGAGGATAAAACGGAGACACAGCTCACAAGAGCAAACC
K V N A E S V E R I K R R H S S Q E Q T
850 870 890
TTCCAGCTGCTGAAGCTGTGGAAACATCAAAACAGAGACCAGGAAATGGTGAAGAAGATC
F Q L L K L W K H Q N R D Q E M V K K I
910 930 950
ATCCAAGACATTGACCTCTGTGAAAGCAGCGTGCAGCGGCATCTCGGCCACTCGAACCTC
I Q D I D L C E S S V Q R H L G H S N L
970 990 1010
ACCACAGAGCAGCTTCTTGCCTTGATGGAGAGCCTGCCTGGGAAGAAGATCAGCCCAGAA
T T E Q L L A L M E S L P G K K I S P E
1030 1050 1070
GAGATTGAGAGAACGAGAAAGACCTGCAAATCGAGCGAGCAGCTCCTGAAGCTACTCAGT
E I E R T R K T C K S S E Q L L K L L S
1090 1110 1130
TTATGGAGGATCAAAAATGGTGACCAAGACACCTTGAAGGGCCTGATGTATGCCCTCAAG
L W R I K N G D Q D T L K G L M Y A L K
1150 1170 1190
CACTTGAAAACATCCCACCTTTCCTCAAACTGTCAACCCACAGTCTGAGGAAGACCATGAGG
H L K T S H F P K T V T H S L R K T M R
1210 1230 1250
TTCCTGCACAGCTTCACAATGTACAGACTGTATCAGAAGCTCTTTTATAGAAATGATAGGG
F L H S F T M Y R L Y Q K L F L E M I G
1270 1290 1310
AATCAGGTTCAATCCGTGAAAATAAGCTGCTTATAACTAGGAATGGTCACTGGGCTGTTT
N Q V Q S V K I S C L
CTTCA

FIG.9C

10 30 50
GTATATATAACGTGATGAGCGTACGGGTGCGGAGACGCACCGGAGCGCTCGCCCAGCCGC
70 90 110
CGYCTCCAAGCCCCTGAGGTTTCCGGGGACCACAATGAACAAGTTGCTGTGCTGCGCGCT
130 150 170
CGTGTTTCTGGACATCTCCATTAAGTGGACCACCCAGGAAACGTTTCCTCCAAAGTACCT
V F L D I S I K W T T O E T F P P K Y L
190 210 230
TCATTATGACGAAGAAACCTCTCATCAGCTGTTGTGTGACAAATGTCCTCCTGGTACCTA
H Y D E E T S H Q L L C D K C P P G T Y
250 270 290
CCTAAAACAACACTGTACAGCAAAGTGAAGACCGTGTGCGCCCCCTTGCCCTGACCACTA
L K Q H C T A K W K T V C A P C P D H Y
310 330 350
CTACACAGACAGCTGGCACACCAGTGACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGA
Y T D S W H T S D E C L Y C S P V C K E
370 390 410
GCTGCAGTACGTCAAGCAGGAGTGCAATCGCACCCACAACCGCGTGTGCGAATGCAAGGA
L Q Y V K Q E C M R T H N R V C E C K E
430 450 470
AGGGCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGCTGCCCTCCTGGATTGG
G R Y L E I E F C L K H R S C P P G F G
490 510 530
AGTGGTGCAAGCTGGAACCCCAGAGCGAAATACAGTTTGCAAAAGATGTCCAGATGGGTT
V V Q A G T P E R N T V C K R C P D G F
550 570 590
CTTCTCAAATGAGACGTCATCTAAAGCACCCCTGTAGAAAACACACAAATTGCAGTGTCTT
F S N E T S S K A P C R K H T N C S V F
610 630 650
TGGTCTCCTGCTAACTCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAG
G L L L T Q K G N A T H D N I C S G N S
670 690 710
TGAATCAACTCAAAAATGTGGAATAGATGTTACCCTGTGTGAGGAGGCATTCTTCAGGTT
E S T Q K C G I D V T L C E E A F F R F
730 750 770
TGCTGTTCTACAAAGTTTACGCCTAAGTGGCTTAGTGTCTTGGTAGACAATTTGCCTGG
A V P T K F T P N W L S V L V D N L P G

FIG. 9D

790 810 830
CACCAAAGTAAACGCAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCACAAGAACA
T K V N A E S V E R I K R Q H S S Q E Q
850 870 890
GACTTTCAGCTGCTGAAGTTATGGAAACATCAAACAAAGACCAAGATATAGTCAAGAA
T F Q L L K L W K H Q N K D Q D I V K K
910 930 950
GATCATCCAAGATATTGACCTCTGTGAAAACAGCGTGCAGCGGCACATTGGACATGCTAA
I I Q D I D L C E N S V Q R H I G H A N
970 990 1010
CCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAAGCTTACCGGGAAAGAAAGTGGGAGC
L T F E Q L R S L M E S L P G K K V G A
1030 1050 1070
AGAAGACATTGAAAAACAATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTGCT
E D I E K T I K A C K P S D Q I L K L L
1090 1110 1130
CAGTTTGTGGCGAATAAAAAATGGCGACCAAGACACCTTGAAGGGCCTAATGCACGCACT
S L W R I K N G D Q D T L K G L M H A L
1150 1170 1190
AAAGCACTCAAAGACGTACCACTTTCCCAAACCTGTCACTCAGAGTCTAAAGAAGACCAT
K H S K T Y H F P K T V T Q S L K K T I
1210 1230 1250
CAGGTTCCCTTCACAGCTTCACAATGTACAAATTGTATCAGAAGTTATTTTATAGAAATGAT
R F L H S F T M Y K L Y Q K L F L E M I
1270 1290 1310
AGGTAACCAGGTCCAATCAGTAAAAATAAGCTGCTTATAACTGGAAATGGCCATTGAGCT
G N Q V Q S V K I S C L
1330 1350
GTTTCCTCACAATTGGCGAGATCCCATGGATGATAA

FIG. 9E

muosteo.frg	MNKKWLCCALLVLLDIIEWTTQETLPPKYYLHYDPEETGHQLLCLCDKCAPGTYL	50
ratosteo.frg	MNKKWLCCALLVLLDIIEWTTQETLPPKYYLHYDPEETGRQLLCLCDKCAPGTYL	50
huosteo.frg	MNKKLLCCALLVFLDISIKWTTQETLPPKYYLHYDEETSHQLLCLCDKCPPGTYL	50
muosteo.frg	KQHCTVRRRKTLCVPCPDHSHSYTDSWHTSDECVYCSPPVCKELQ	100
ratosteo.frg	KQHCTVRRRKTLCVPCPDHSHSYTDSWHTSDECVYCSPPVCKELQ	100
huosteo.frg	KQHCTARWKTVCAPCPDHYTYTDSWHTSDECLYCSPPVCKELO	100
muosteo.frg	HNRVCECEEEGRYLEIEFCLKHRSCPPPGSGVYVQAGTPERNNTVCKKCPDGGFF	150
ratosteo.frg	HNRVCECEEEGRYLELEFCLKHRSCPPPGGLGVLLQAGTPERNNTVCKRCPDGGFF	150
huosteo.frg	HNRVCECKEGRYLEIEFCLKHRSCPPPGFGVYVQAGTPERNNTVCKRCPDGGFF	150
muosteo.frg	SGETSSSKAPCIKHTNCS	200
ratosteo.frg	SGETSSSKAPCRKHTNCS	200
huosteo.frg	SNLETSSSKAPCRKHTNCS	200
	TFGLLLIQKGNATHDNVCSGNSGNREATAQKCGIDVT	
	SLGLLLIQKGNATHDNVCSGNSGNREATAQKCGIDVT	
	VFGLLLTQKGNATHDNVCSGNSGNSESTQKCGIDVT	

FIG. 9F

muosteo.frg	L	C	E	E	A	F	F	R	F	A	V	P	T	K	I	I	P	N	W	L	S	V	L	V	D	S	L	P	G	T	K	V	N	A	E	S	V	E	R	I	K	R	R	H	S	S	Q	E	Q	T	250	
ratosteo.frg	L	C	E	E	A	F	F	R	F	A	V	P	T	K	I	I	P	N	W	L	S	V	L	V	D	S	L	P	G	T	K	V	N	A	E	S	V	E	R	I	K	R	R	H	S	S	Q	E	Q	T	250	
huosteo.frg	L	C	E	E	A	F	F	R	F	A	V	P	T	K	I	F	T	P	N	W	L	S	V	L	V	D	N	L	P	G	T	K	V	N	A	E	S	V	E	R	I	K	R	Q	H	S	S	Q	E	Q	T	250

muosteo. frg	F Q L L K L W K K H Q N R R D Q E M V K K I I Q D I D L C E S S V Q R H L G H S N L T T E Q L L A L M E	300
atosteo. frg	F Q L L K L W K K H Q N R R D Q E M V K K I I Q D I D L C E S S V Q R H I G H A N L T T E Q L R I L M E	300
huosteo. frg	F Q L L K L W K K H Q N K D Q D I V K K I I Q D I D L C E N S V Q R H I G H A N L T F E Q L R S L M E	300

muosteo.frg	S	L	P	G	K	K	I	S	P	E	E	I	E	R	T	R	K	T	C	K	S	S	E	Q	L	L	K	L	L	S	L	W	R	I	K	N	G	D	Q	D	T	L	K	G	L	M	Y	A	L	K
ratosteo.frg	S	L	P	G	K	K	I	S	P	D	E	I	E	R	T	R	K	T	C	K	P	S	E	Q	L	L	K	L	L	S	L	W	R	I	K	N	G	D	Q	D	T	L	K	G	L	M	Y	A	L	K
huosteo.frg	S	L	P	G	K	K	V	G	A	E	D	I	E	K	T	I	K	A	C	K	P	S	D	Q	L	L	K	L	L	S	L	W	R	I	K	N	G	D	Q	D	T	L	K	G	L	M	H	A	L	K

mmosteo. frg	H	L	K	T	S	H	F	P	K	T	V	T	H	S	L	R	K	T	M	R	F	L	H	S	F	T	M	Y	R	L	Y	Q	K	L	F	L	E	M	I	G	N	Q	V	Q	S	V	K	I	S	C	400
ratosteo. frg	H	L	K	A	Y	H	F	P	K	T	V	T	H	S	L	R	K	T	I	R	F	L	H	S	F	T	M	Y	R	L	Y	Q	K	L	F	L	E	M	I	G	N	Q	V	Q	S	V	K	I	S	C	400
huosteo. frg	H	S	K	T	Y	H	F	P	K	T	V	T	Q	S	L	K	K	T	I	R	F	L	H	S	F	T	M	Y	R	L	Y	Q	K	L	F	L	E	M	I	G	N	Q	V	Q	S	V	K	I	S	C	400

muosteo.frg L 401
ratosteo.frg L 401
huosteo.frg L 401

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FIG.10

ltnrr	C	P	Q	-	G	K	Y	I	H	P	Q	N	N	S	I	C	C	T	K	C	H	K	G	T	Y	L	Y	N	D	C	P	G	P	Q	D	T	D	C	R	E	C	E	S	G	S	F	T	A	S	49	
humoste	P	P	K	Y	L	H	Y	D	E	E	T	S	H	Q	L	L	C	D	K	C	P	P	G	T	Y	L	K	Q	H	C	T	A	K	-	W	K	T	V	C	A	P	C	P	D	H	Y	Y	T	D	S	49
ltnrr	E	N	H	L	R	H	C	L	S	C	S	-	K	C	R	K	E	M	G	Q	V	E	I	S	S	C	T	V	D	R	D	T	V	C	G	C	R	K	N	Q	Y	R	H	Y	W	S	E	N	L	F	98
humoste	W	H	T	S	D	E	C	L	Y	C	S	P	V	C	-	K	E	L	Q	Y	V	K	-	Q	E	C	N	R	T	H	N	R	V	C	E	C	K	E	G	R	Y	L	E	I	-	-	E	-	F	93	
ltnrr	Q	C	F	N	C	S	L	C	L	N	G	-	T	V	H	L	S	C	Q	E	K	Q	N	T	V	C	T	-	C	H	A	G	F	F	L	R	E	-	-	-	N	E	C	V	S	C	139				
humoste	-	C	F	K	H	R	S	C	P	P	G	F	G	V	V	Q	A	G	T	P	E	R	N	T	V	C	K	R	C	P	D	G	F	F	S	N	E	T	S	S	K	A	P	C	R	K	H	139			

FIG. II

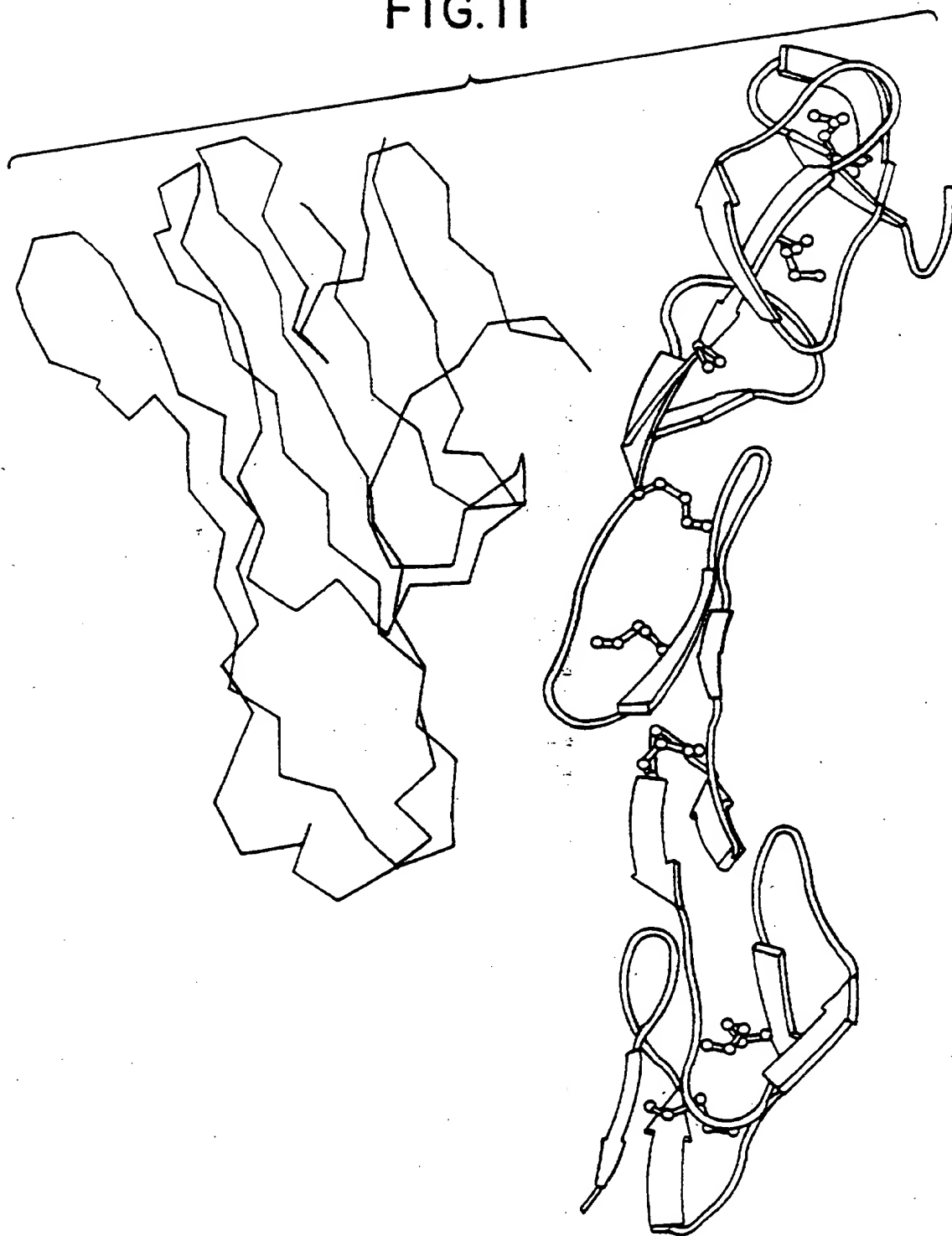
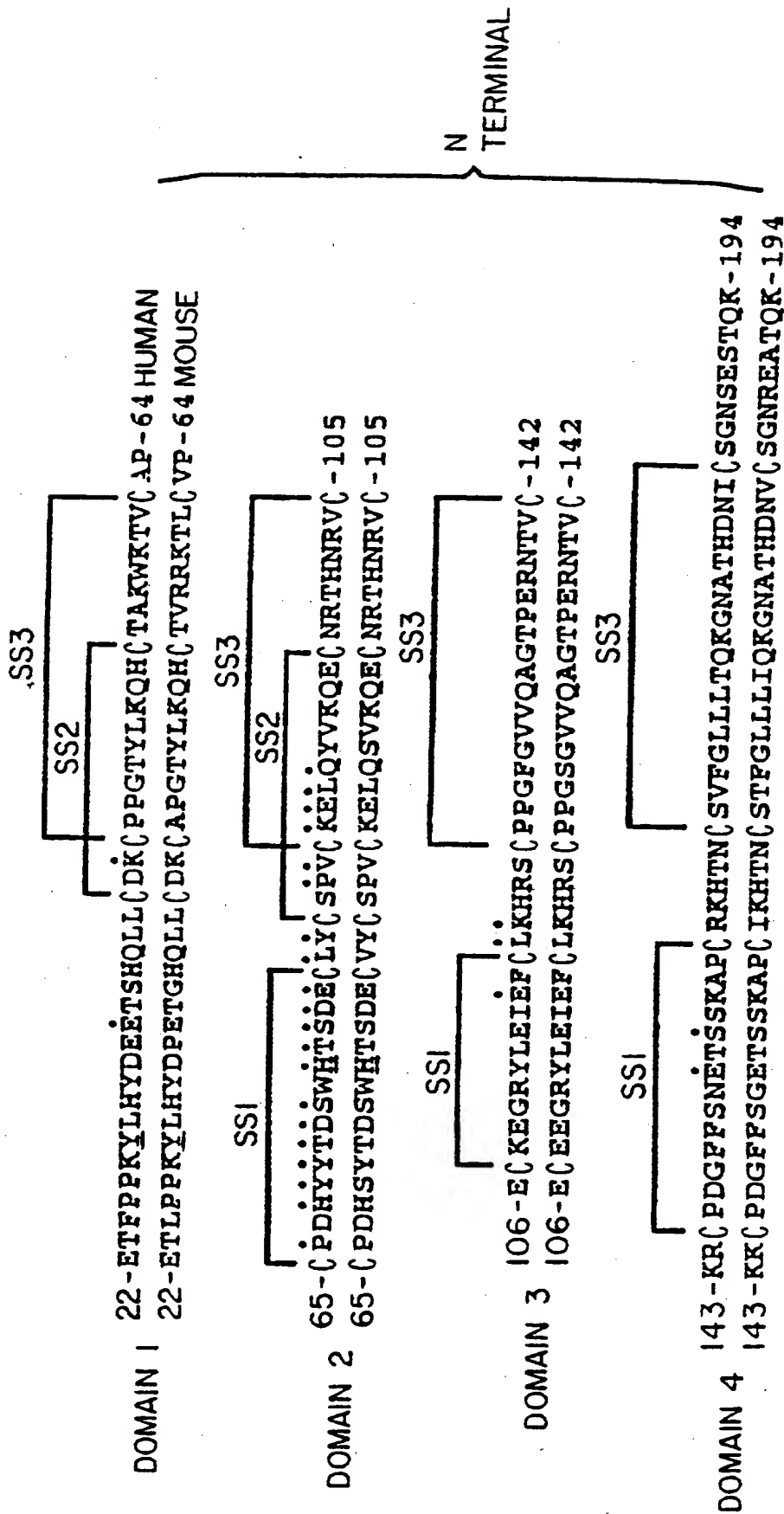


FIG.12A



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FIG. 12B

195-CGIDVTLCEEAFPRFAVPTKFTPNWLSVLVDNLPGTKVNAESVERIKRQHSS-246
195-CGIDVTLCEEAFPRFAVPTKFTPNWLSVLVDNLPGTKVNAESVERIKRRHSS-246
247-QEQTFO¹LLKWKHQNKQDQDI²VKKIIQDID³LC⁴ENSVQRHGHANLTPEQLRSL-298
247-QEQTFO¹LLKWKHQNRDQEMVKKIIQDID³LC⁴ESSVQRHLGHSNLTTEQLLAL-298
299-MESLP⁵GKKVGAEDIEKTIK⁶CP⁷SDQILKLLSLWRIKNGDQDTLKGLMHALK-350
299-MESLP⁵GKKISPEEIERTRKT⁶CK⁷SEQLKLLSLWRIKNGDQDTLKGLMYALK-350
351-HSKTYHFPKTVTQSLKKTIRFLHSFTMYKLYQKLFLEMIGNQVQSVKIS⁸CL-401
351-HLKTSHFPKTVTTHSLRKTMRFLHSFTMYRLYQKLFLEMIGNQVQSVKIS⁸CL-401

C } TERMINAL

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FIG.13A

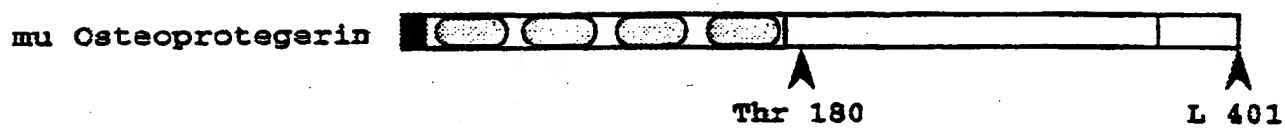


FIG.13B

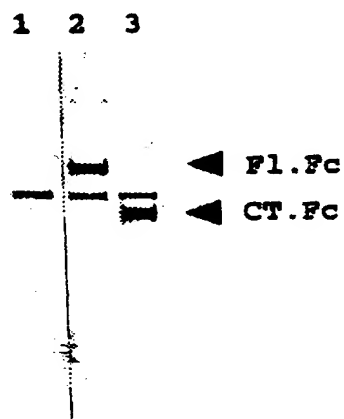
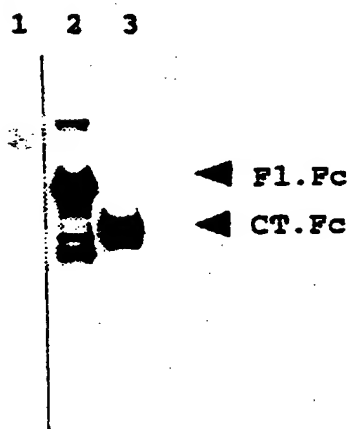
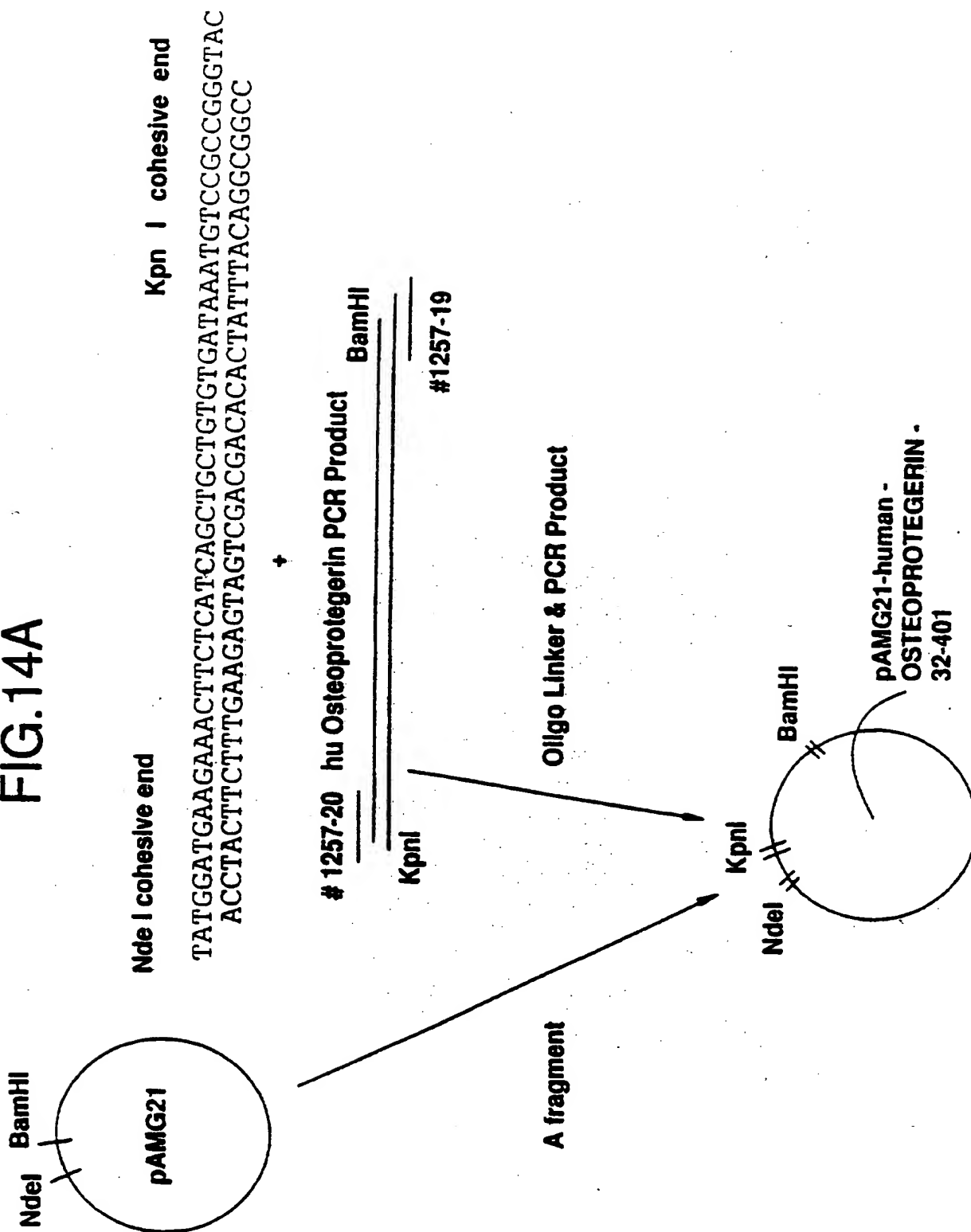


FIG.13C



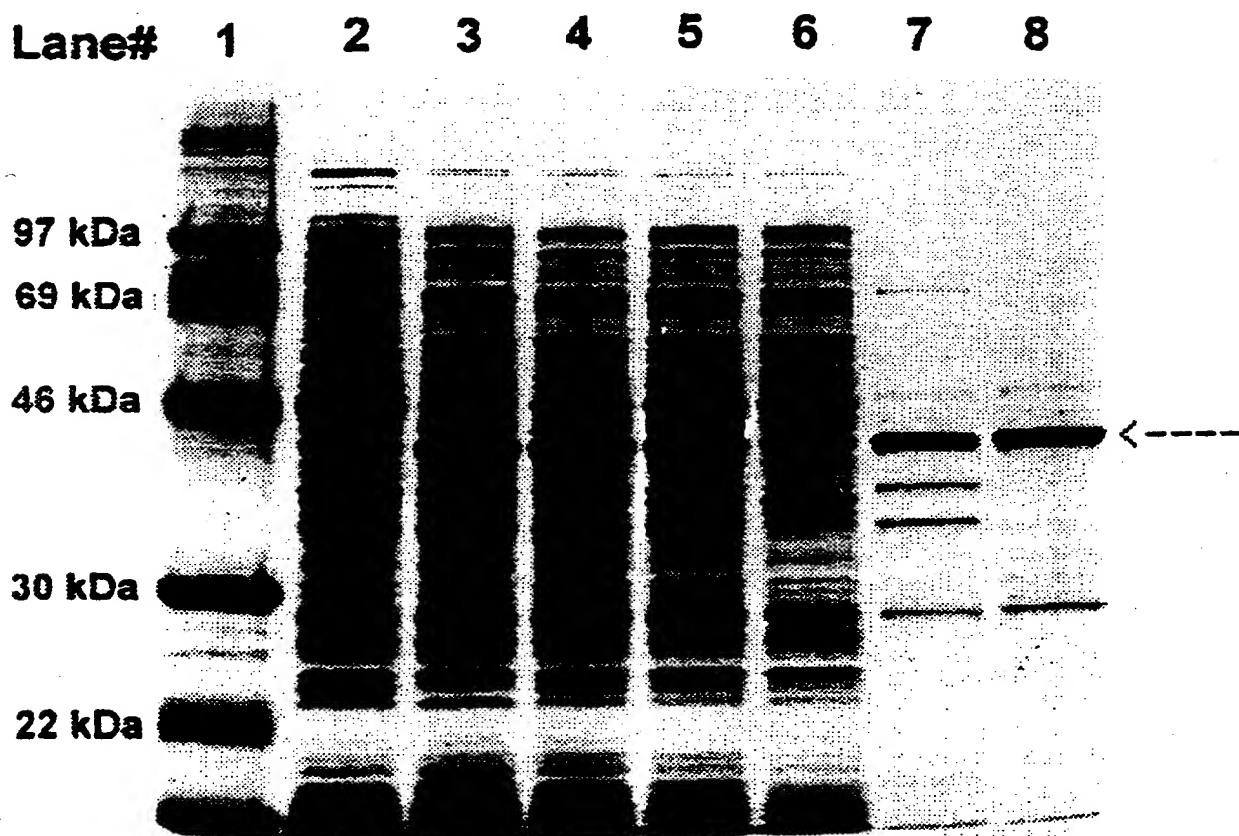
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FIG.14A



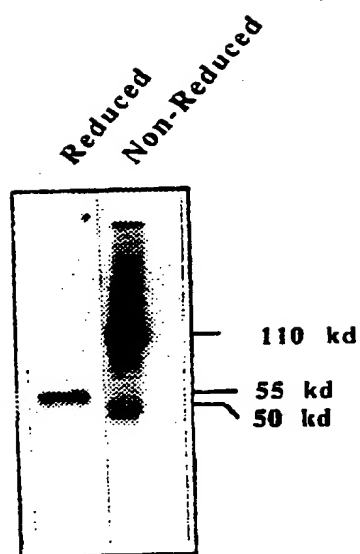
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FIG.14B



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FIG.15



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FIG. 16A

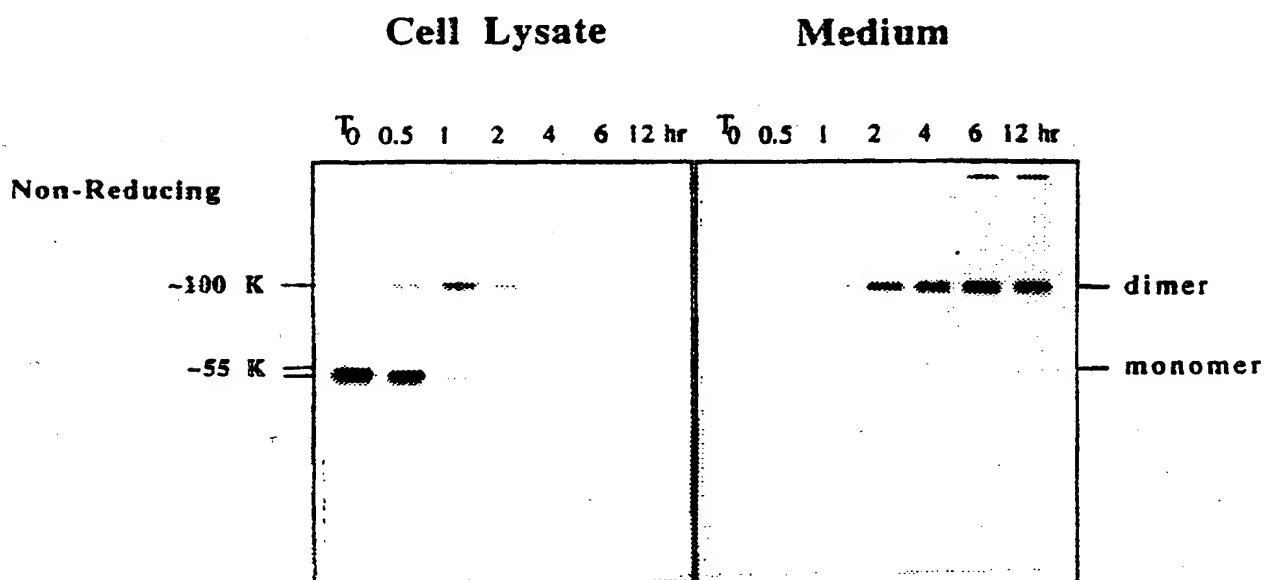
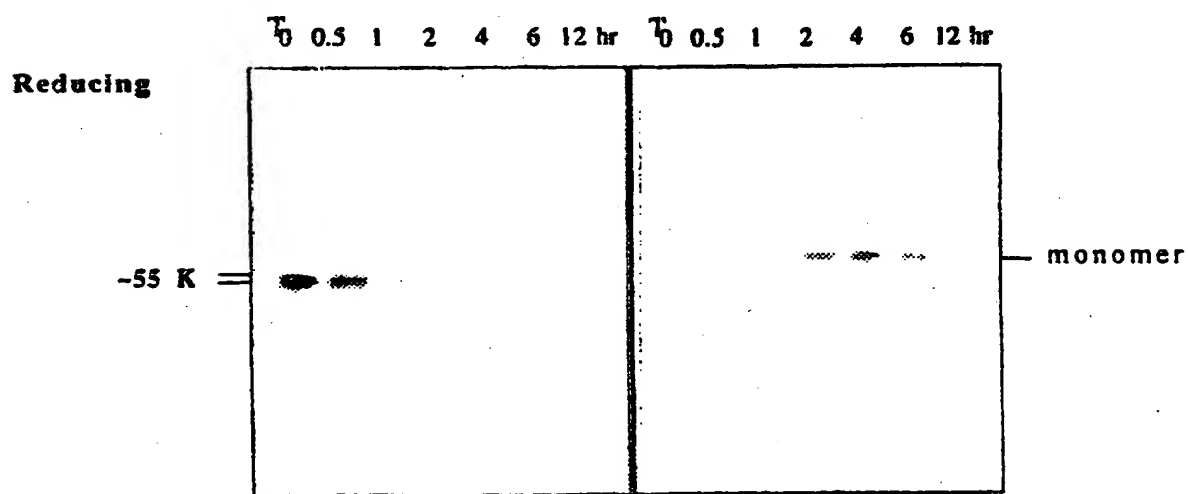
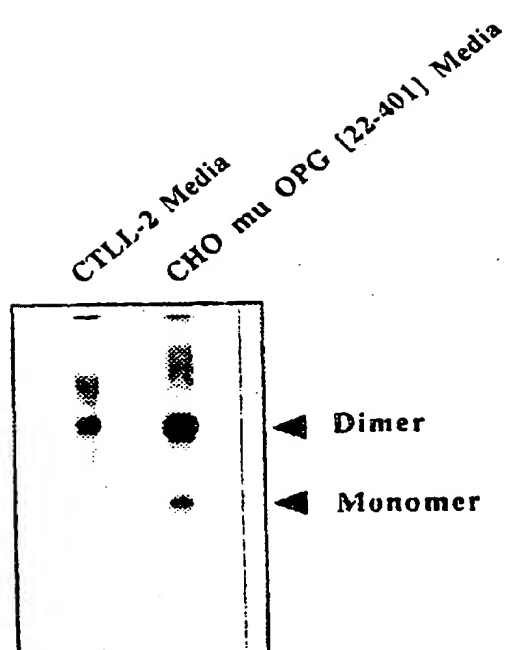


FIG. 16B



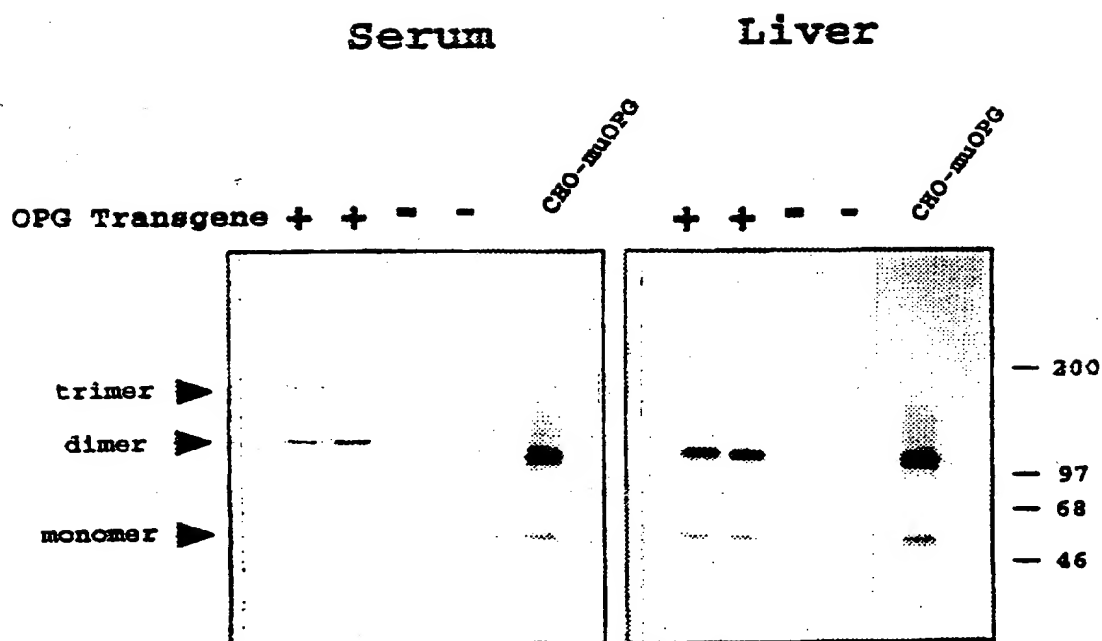
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FIG. 17



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FIG.18



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FIG.19A

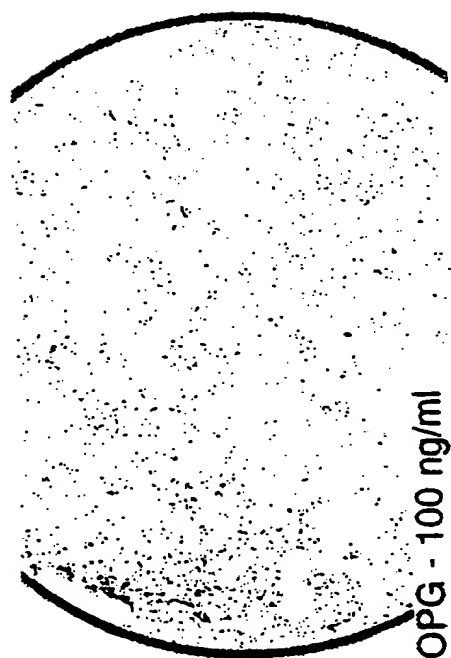


FIG.19B

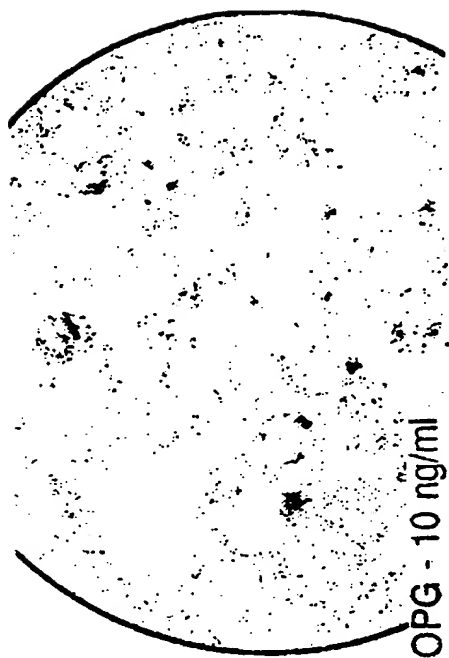


FIG.19C

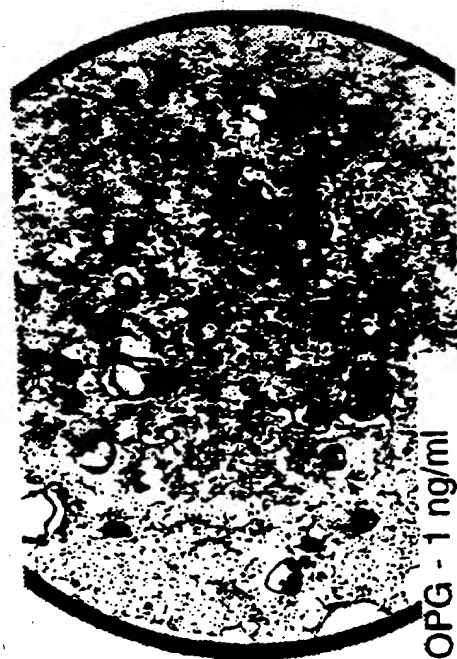
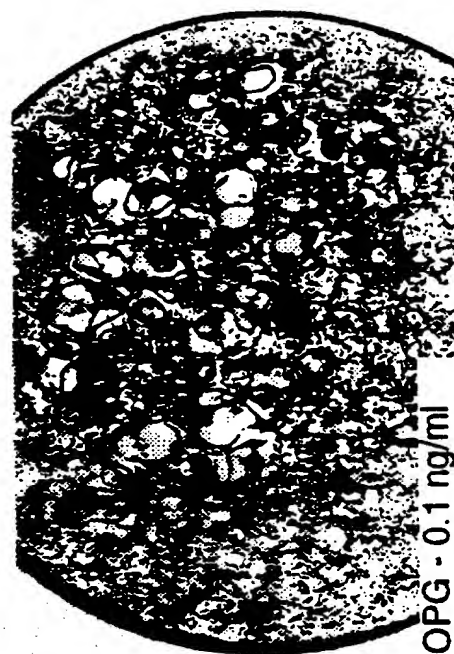


FIG.19D



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FIG.19F

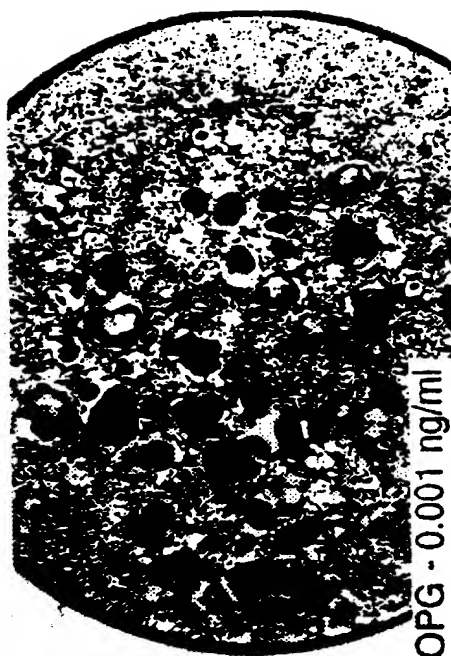


FIG.19E

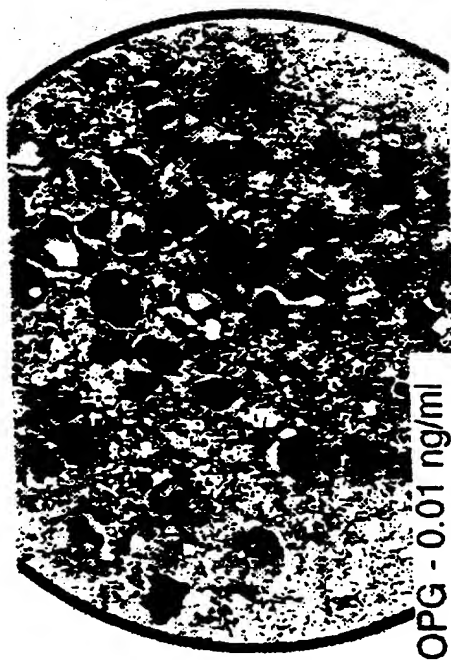
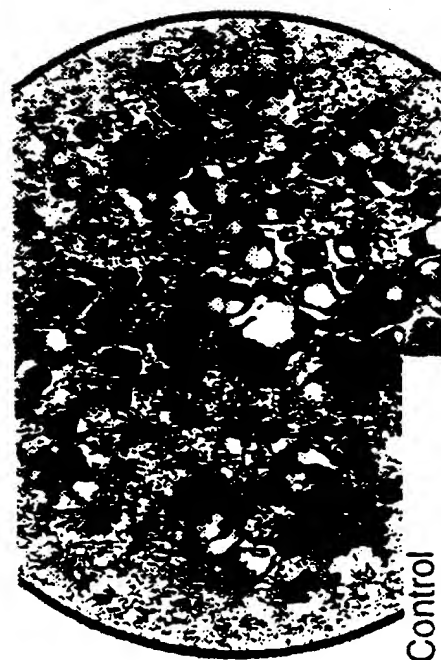


FIG.19G



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FIG.20

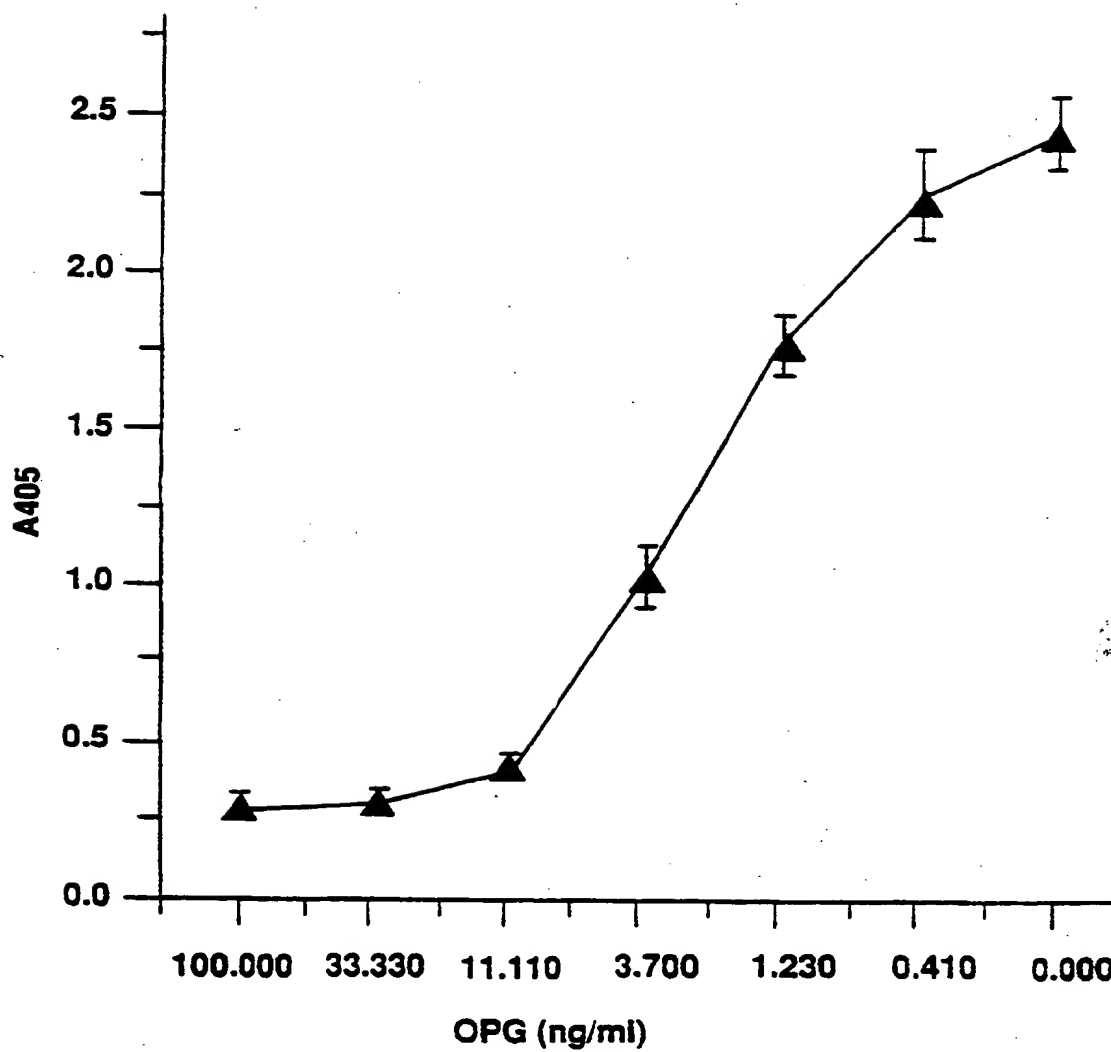
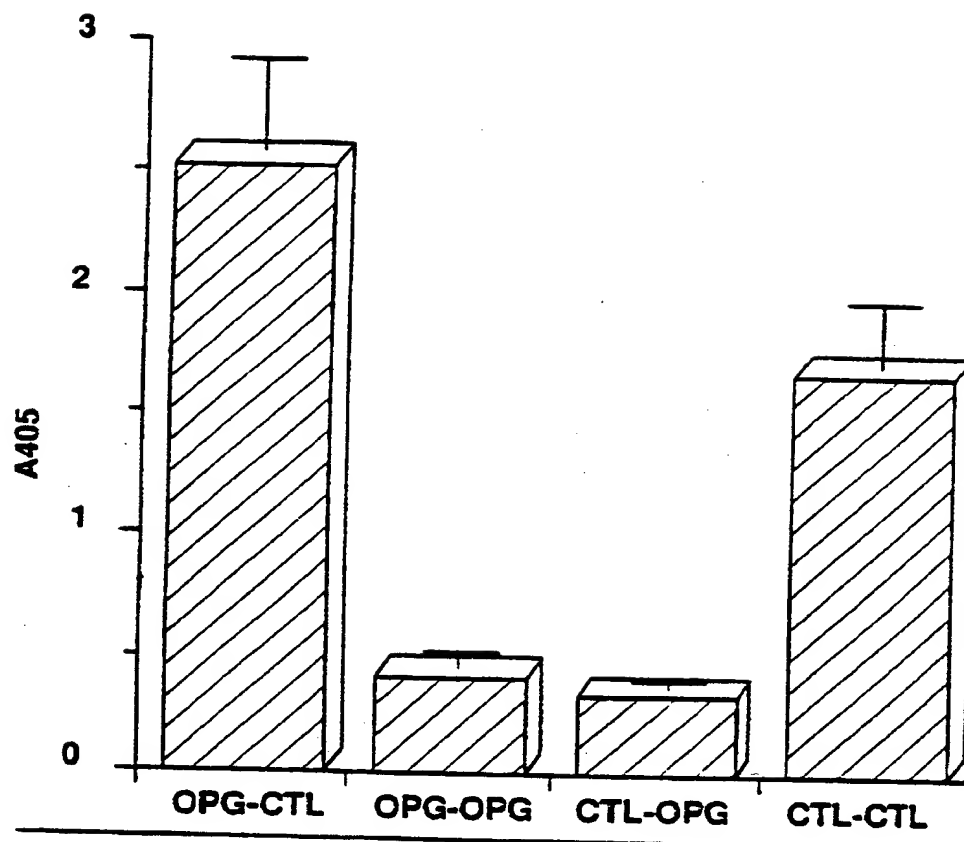


FIG.21



Legend

Growth
Bone marrow
cells
CSF -1

Intermediate
PGE2 + CSF-1

Terminal
ST2 cells
1,25 (OH)2 D3
Dexamethasone

4 days

2 days

8 - 10 days

Groups

CTL - CTL

OPG - CTL

OPG - OPG

OPG - OPG

OPG

100 ng/ml

100 ng/ml

OPG

100 ng/ml

100 ng/ml

FIG.22A

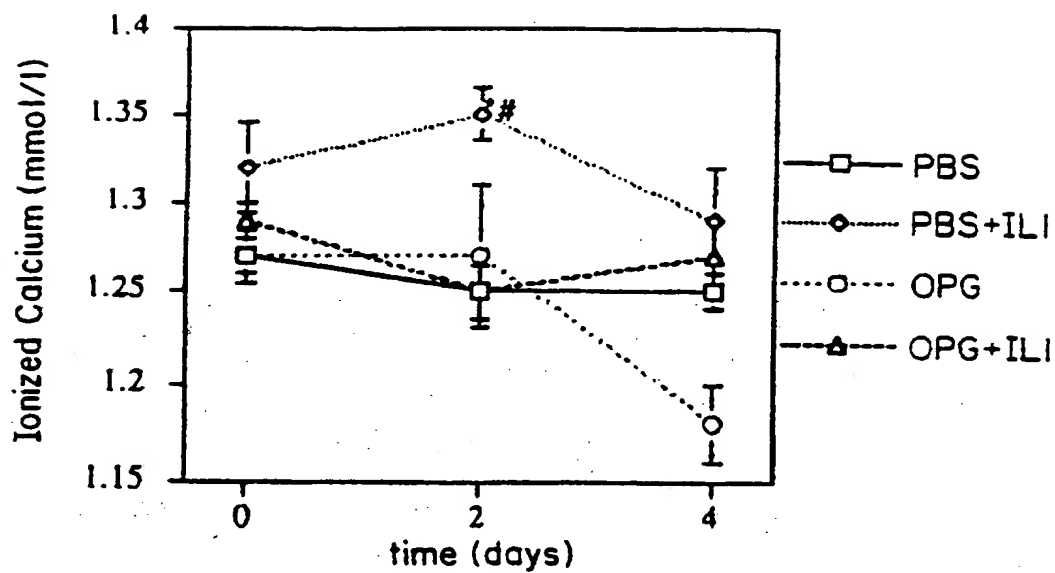
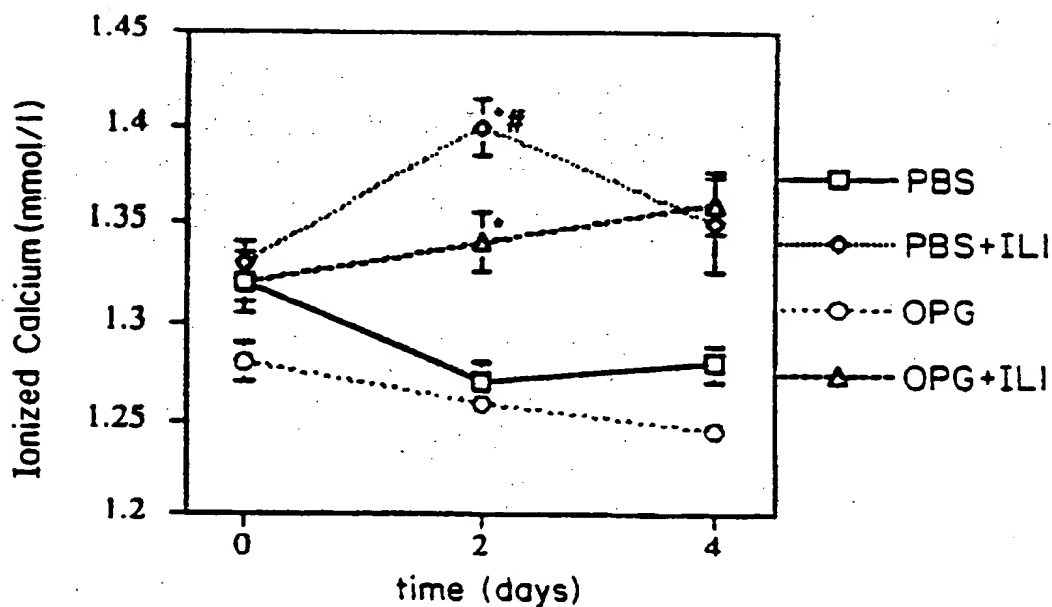


FIG.22B



* Different to PBS, $p < 0.05$

Different to OPG + IL1, $p < 0.05$

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FIG.23A

PBS/PBS



FIG.23B



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FIG.23C

PBS/OPG

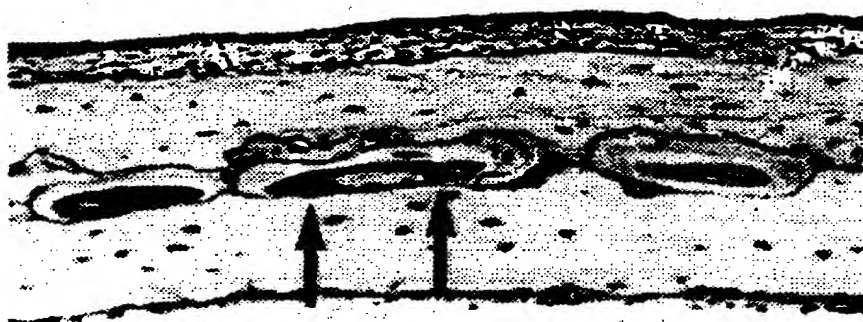
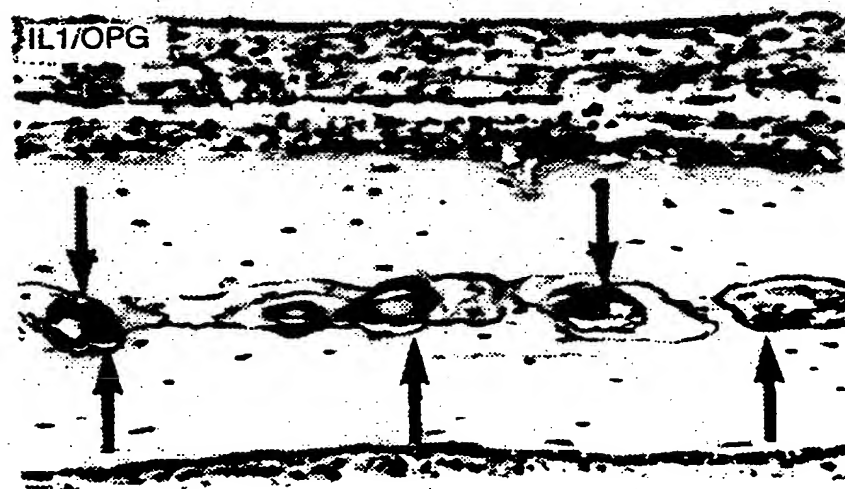


FIG.23D



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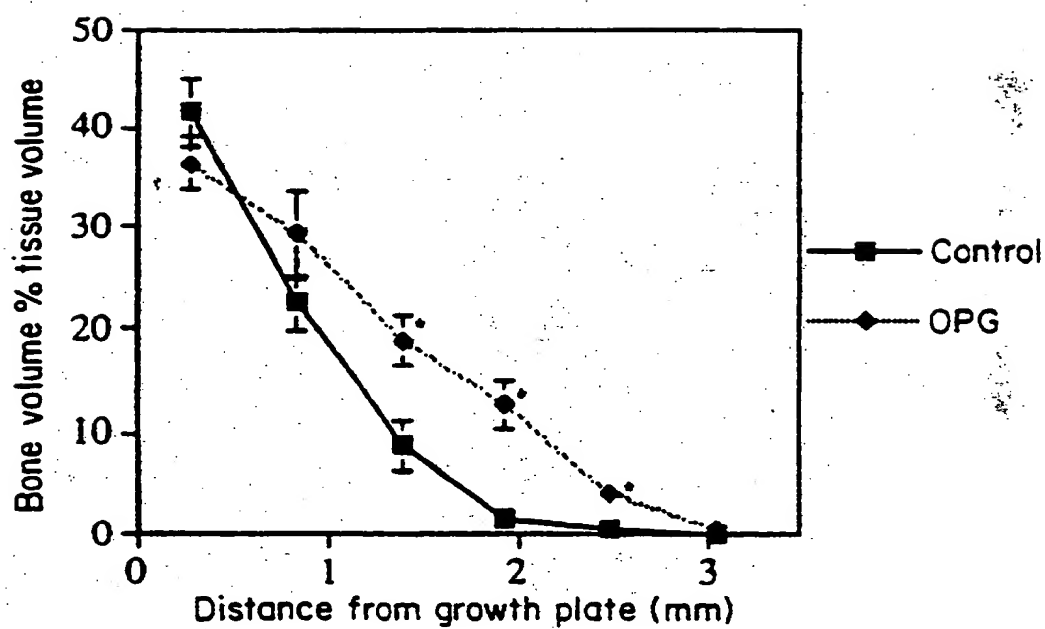
FIG. 24B



FIG. 24A



FIG. 25



* Different to control $p < 0.01$

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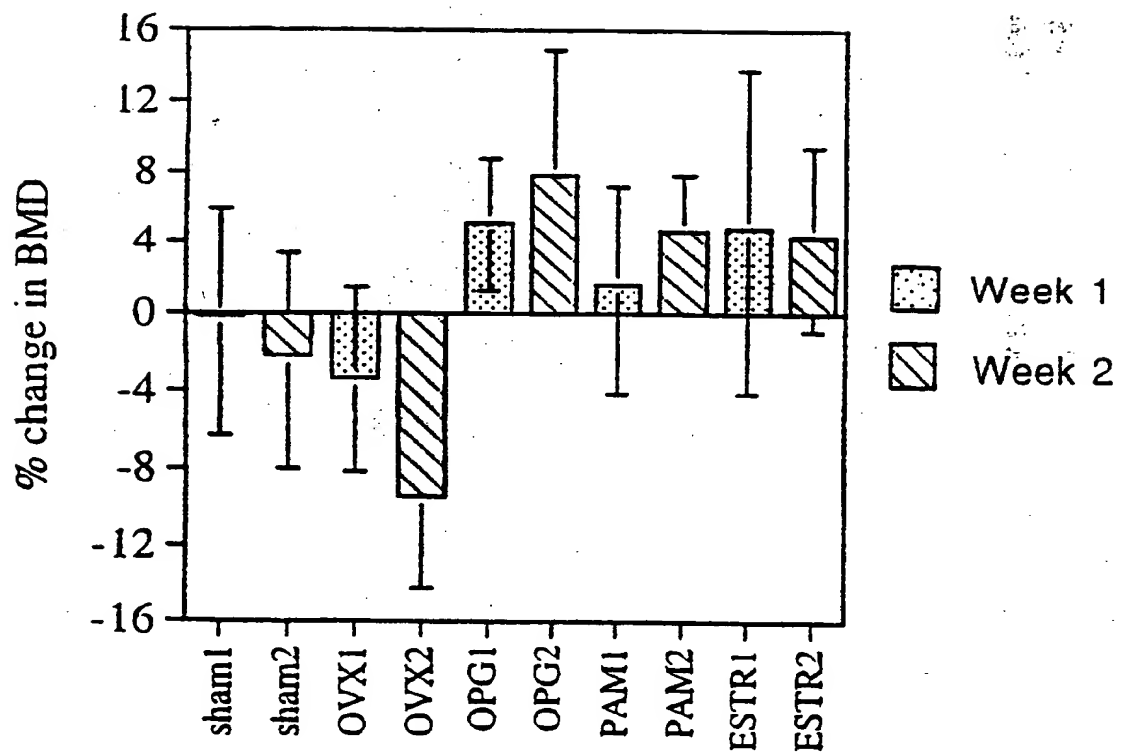
FIG.26A



FIG.26.B

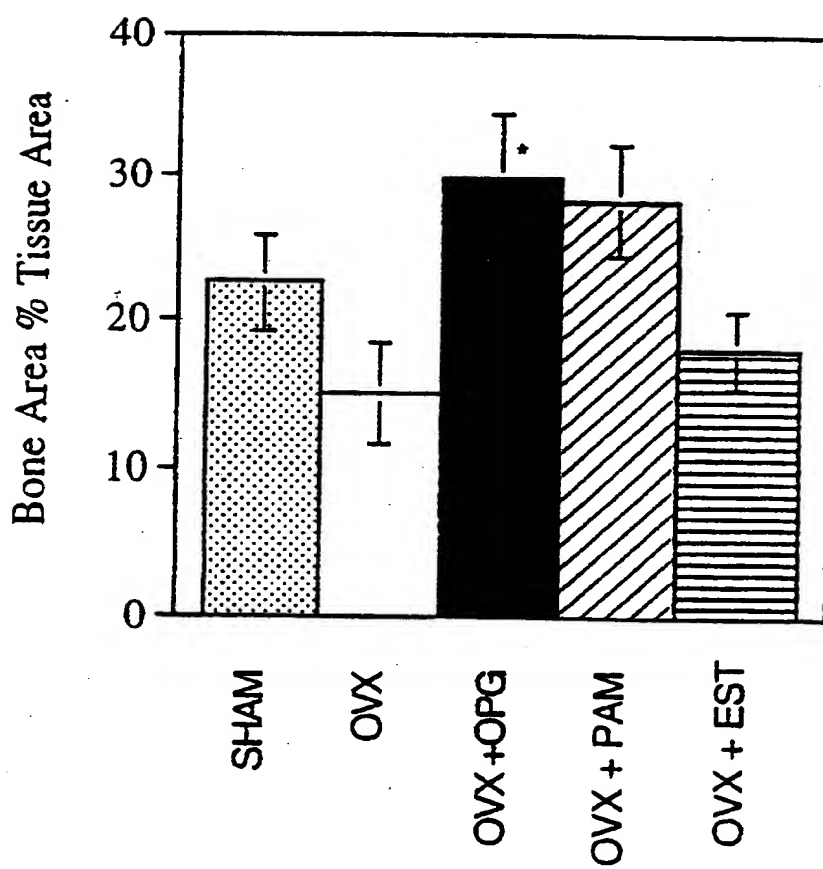


FIG. 27



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FIG.28



* Different to OVX $p < 0.05$

INTERNATIONAL SEARCH REPORT

Internat Application No
PC1/US 96/20621

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/12 C07K14/715 C12N5/10 A01K67/027 C07K19/00
 C12N15/62 C07K16/28 C07K1/107 C12Q1/68 G01N33/50
 G01N33/56 A61K38/17 A61K48/00 C12N1/21 //(C12N1/21,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A01K C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL, vol. 76, 25 March 1994, pages 959-962, XP002029050 SMITH C.A. ET AL.: "The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death." cited in the application see the whole document ---	1-60
A	PROC.NATL.ACAD.SCI.USA, vol. 88, 1991, pages 2830-2834, XP002029051 LEWIS M. ET AL.: "Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific." see figure 1 --- -/-	1-60

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

9 April 1997

Date of mailing of the international search report

16. 04. 97

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 Fax (+31-70) 340-3016

Authorized officer

Mandl, B

INTERNATIONAL SEARCH REPORT

International Application No.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol. 252, 1991, pages 1651-1656, XP000645049 ADAMS M.D.: "Complementary DNA sequencing: expressed sequence tags and human genome project." cited in the application see the whole document ---	1-60
A	US 5 447 851 A (BEUTLER BRUCE A ET AL) 5 September 1995 see the whole document ---	36-38,57
A	US 4 179 337 A (DAVIS FRANK F ET AL) 18 December 1979 cited in the application see the whole document -----	28,29

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☐ Patent family members are listed in annex.

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- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/20621

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 43-45, 49-53
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/20621

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5447851 A	05-09-95	NONE	
US 4179337 A	18-12-79	CA 1033673 A	27-06-78
		CH 616942 A	30-04-80
		DE 2433883 A	05-02-76
		FR 2313939 A	07-01-77
		GB 1469472 A	06-04-77
		JP 1152589 C	30-06-83
		JP 50042087 A	16-04-75
		JP 56023587 B	01-06-81
		NL 7409770 A	22-01-75
		SE 441753 B	04-11-85
		SE 7409366 A	21-01-75